Ingénierie et aspects microbiens du procédé Anammox pour le traitement des eaux usées

Zhiji Ding

Engineering and microbial aspects of Anammox process in wastewater treatment

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Zhiji Ding (October, 2015)

Summary

To meet the requirements of nitrogen limit in the effluent from the wastewater treatment plant (WWTP), biological nitrogen removal has become the mainstream process thanks to its high removal efficiency. The incorporation of nitrification/denitrification pathway in the conventional activated sludge process has achieved the most widely full-scale application for municipal and industrial wastewaters since 1960s. Whereas in recent years, the discovery of anaerobic Ammonium oxidation (Anammox) process offered an alternative pathway to remove ammonium from wastewater, in which ammonium was anaerobically oxidized into nitrogen gas with nitrite as electron acceptor by autotrophic bacteria. The benefit of lower oxygen demand, no necessity of extra carbon source and lower ecological footprint made this an attractive option as biological wastewater treatment. The scope of the study is therefore "*to investigate the process of Anammox enrichment and evolution of microbial diversity and EPS characteristics for treating wastewater with low C/N ratio*".

Due to its extremely slow growth rate and scarcity in enriched sludge, full-scale application of the Anammox process is hindered. Therefore, enrichment process of Anammox biomass from three different types of conventional seeding sludge was studied, including aerobic sludge, denitrification sludge and anaerobic floccular sludge. The effects of oxygen control and selection pressure were investigated. Anammox process was successfully established in the reactor seeded with denitrification sludge with a total nitrogen removal of approximately80% under strict oxygen control after 150 days. The enrichment was confirmed by monitoring the chemical composition of the influent/effluent and the evolution of the microbial diversity was evidenced by denaturing gradient gel electrophoresis (DGGE) fingerprint after polymerase chain reaction (PCR). With a shorter hydraulic retention time (HRT) and no strict oxygen control, the reactor seeded with aerobic sludge reached 50-60% total nitrogen removal after 240 days. Reactors seeded with anaerobic sludge could not establish desirable Anammox process, despite two trials with different operation control. All the reactors experienced fluctuating performances during the enrichment process, which is believed to be the consequence of inhibitory factors such as dissolved oxygen, free nitrite and free ammonia as well as undesirable coexisting bacteria which compete for the same substrate. A clear evolution of the microbial composition was evidenced bythe similarity of DGGE band from the amplified DNA samples extracted from different enrichment stage of the reactor seeded with aerobic sludge.

Extracellular polymeric substances (EPS) are believed to play important role in bioaggregation and have high correlation with Anammox enrichment process. Evolution of EPS characteristics was investigated through a series of quantitative and qualitative analysis. An increase in total EPS extraction yield and a decrease of protein to polysaccharide (PN/PS) ratio and an increase in total EPS extraction yield were observed during the enrichment process. The three dimensional excitation emission matrix (3D-EEM) showed similar location of the fluorescence peaks for all EPS samples while samples extracted from the sludge containing higher amount of Anammox bacteria possessed two distinct peaks in the low excitation wavelength range. During enrichment, ultraviolet (UV) size exclusion chromatographic fingerprint showed increase in both intensity and number of peaks, whereas protein-like fluorescence chromatograms showed similar peak number and only increase in intensity. An increase of EPS hydrophobicity was observed during the enrichment process.

To conclude, the coverage of the work was positioned in the framework of microbial resource management (MRM), which is composed of "engineer's input", "microbial black box" and "process output". In this thesis, a series of studies on "engineer's input", including the enrichment of Anammox biomass and test of one-reactor partial nitritation/Anammox process, have been conducted aiming at the main MRM output of "high nitrogen removal" and "accelerated enrichment". To reveal the dynamics of the "microbial black box", molecular technique PCR-DGGE was performed on different time of the enrichment period. Quantitative and qualitative EPS analysis was conducted aiming at establishing the correlation between EPS characteristics and process parameters.

Samenvattig (Dutch)

Biologische stikstofverwijdering is uitgegroeid tot één van de belangrijkste zuiveringsprocessen van afvalwaterzuiverings installaties, vanwege de hoge verwijderings efficiëntie van het proces. De integratie van nitrificatie en denitrificatie in het conventionele actief slib proces is de meest succesvolle applicatie voor gemeentelijk en industriëel afvalwater sinds de jaren 1960. De ontdekking van het anaërobe ammonium oxidatie (Anammox) proces biedt een alternatief proces om ammonium uit afvalwater te verwijderen. In dit proces wordt ammonium anaëroob geoxideerd tot stikstofgas door autotrofe bacteriën met nitriet als elektronen acceptor. De lagere zuurstofvraag, geen behoefte aan extra koolstofbronnen en de lagere ecologische voetafdruk maken dit proces een aantrekkelijke optie om afvalwater biologisch te zuiveren. Het doel van deze studie was om het Anammox proces voor de behandeling van afvalwater met een lage C / N-verhouding te onderzoeken, en met name de aanrijking en de evolutie van de microbiële diversiteit, alsook de eigenschappen van EPS.

De industriële toepassing van het Anammox-proces wordt belemmerd door de trage groei van dit microorganisme en schaarste van verrijkt slib. Daarom wordt in deze studie het verrijkingsproces van Anammox biomassa in drie verschillende types conventioneel slib, met name aëroobslib, denitrificerendsliben anaëroob flocculant slib, onderzocht. Het effect van zuurstof en selektiedruk werd onderzocht. Het Anammox proces werd succesvol geïmplementeerd na 150 dagenin een reactor met denitrificerend slib met een totale stikstofverwijdering van ongeveer 80% onder strikte zuurstofcontrole. De verrijking werd bevestigd door het opvolgen van de chemische samenstelling van het in- en effluent. Tevens werd de evolutie van de microbiële diversiteit aangetoond door een denaturerende gradiënt gelelektroforese (DGGE) vingerafdruk na een polymerase-kettingreactie (PCR). Met een kortere hydraulische retentietijd (HRT) en geen strikte zuurstof controle, werd in de reactor geïnoculeerd met aëroob slib een totale stikstofverwijdering van 50-60% bekomen na 240 dagen. Reactoren geïnoculeerd met anaëroob slib waren niet in staat het Anammox-proces te stabiliseren, ondanks twee proeven met verschillende operationelecontroles. Alle reactoren hadden een fluctuerende performantie gedurende het verrijkingsproces. Er wordt aangenomen dat dit het gevolg is van remmende factoren zoals de opgeloste zuurstof, vrij nitriet en vrij ammoniak, alsook door ongewenste bacteriën die concurreren voor dezelfde substraten. Een duidelijke evolutie van de microbiële samenstelling werd aangetoond door een DGGE-

analyse van de geamplificeerde DNA-monsters geëxtraheerd uit verschillende verrijkingsstappen van de reactor geënt met aëroob slib.

Extracellulaire polymere stoffen (EPS) worden verondersteld een belangrijke rol te spelen bij aggregatie en hebben een nauw verband met het Anammox verrijkingsproces. Evolutie van de EPS kenmerken werden onderzocht door middel van een reeks kwantitatieve en kwalitatieve analyses. Een toename van de totale EPS opbrengst en een afname van eiwit/polysaccharide (PN/PS) verhouding werden tijdens het verrijkingsproceswaargenomen. De driedimensionale excitatie emissie matrix (3D-EEM) toonde soortgelijke locaties van de fluorescentie pieken voor alle EPS monsters. Voor slib met Anammox-bacteriën werden twee afzonderlijke pieken in de lage excitatie golflengtes gevonden. Tijdens verrijking toonde ultraviolet (UV) deeltjesgrootte uitsluiting chromatografie een stijging van zowel de intensiteit als het aantal pieken, terwijl eiwit fluorescentie chromatogrammen enkel een verschil in intensiteit vertoonden. Een toename van de EPS hydrofobiciteit werd waargenomen tijdens het verrijkingsproces.

Het onderzoek werd geplaatst in het kader van microbieel resource management (MRM), welke is samengesteld uit een "ingenieur input", een "microbiële black box" en een "proces output". In dit proefschrift werden een aantal studies over de "ingenieur input" uitgevoerd met het oog op de belangrijkste MRM output van "hoge stikstofverwijdering" en "versnelde verrijking", met name verrijking van de Anammox biomassa en het testen van een partieel nitritatie/Anammox proces. Om de dynamiek van de “microbiële black box” in kaart te brengen, werd de moleculaire techniek PCR-DGGE uitgevoerd op verschillende tijdstippen van de verrijkingsperiode. Een kwantitatieve en kwalitatieve EPS analyse werd uitgevoerd met het oog op de correlatie tussen EPS karakteristieken en procesparameters.

Sommario (Italian)

Grazie all'elevata efficienza che lo caratterizza, il processo di rimozione biologica dell'azoto si è affermato come sistema più impiegato per assicurare il rispetto dei limiti normativi sull'azoto contenuto nei reflui depurati dagli impianti di trattamento della acque. Fin dagli anni 60, infatti, l'inserimento di una fase nitro/denitro a completamento del processo convenzionale a fanghi attivi ha rappresentato la soluzione più largamente utilizzata negli impianti di trattamento per reflui civili e industriali. In anni recenti, però, la scoperta dell'ossidazione dell'ammonio in ambiente anaerobico (Anammox) in cui l'ammonio è anaerobicamente ossidato ad azoto gas da batteri autotrofi che utilizzano nitriti come accettori di elettroni, ha mostrato una soluzione alternativa alla nitro/denitro. I vantaggi derivanti dall'uso di questo processo, tra cui una minore impronta ecologica e una richiesta minore di ossigeno e nulla di una fonte organica di carbonio, lo rendono molto conveniente per il trattamento biologico delle acque reflue. Pertanto, scopo di questo lavoro di tesi è stato "studiare il processo di arricchimento della biomassa Anammox e la sua evoluzione microbica e in termini di EPS (sostanze polimeriche extracellulari) impiegata nel trattamento di acque reflue con un basso rapporto C/N".

L'estrema lentezza con cui la biomassa Anammox cresce e la l'esigua presenza in fanghi già arricchiti, hanno ostacolato il diffondersi del processo Anammox per il trattamento dei reflui in impianti a scala reale. Pertanto nel presente lavoro di tesi è stato studiato il processo di arricchimento della biomassa Anammox in tre differenti tipi di fanghi convenzionali, quali fango attivo, fango di un processo di denitrificazione, fango anaerobico. In particolare sono stati esaminati gli effetti della somministrazione controllata dell'ossigeno e di varie strategie di selezione microbica. I risultati hanno mostrato che la biomassa Anammox si è sviluppata con successo nel reattore inoculato con fango proveniente da un processo di denitrificazione raggiungendo un'efficienza di rimozione dell'azoto dopo 150 giorni di funzionamento e sotto un controllo rigido sulla presenza di ossigeno di circa 80%. Il processo di arricchimento del fango è stato confermato dal confronto della composizione chimica dell'effluente e dell'influente e l'evoluzione delle famiglie microbiche nel fango è stato evidenziato dall'impronta dell'elettroforesi su gel in gradiente denaturante (DGGE), dopo reazione a catena della polimerasi (PCR). Il reattore invece, inoculato con fango attivo e operante con un minore tempo di detenzione idraulico e senza un rigido controllo sulla presenza di ossigeno ha raggiunto un'efficienza di rimozione dell'azoto totale del 50-60% dopo 240 giorni di

funzionamento. I reattori inoculati con fango anerobico, invece, non hanno mostrato lo sviluppo di un'adeguata biomassa Anammox, nonostante sono stati portati a termine due tentativi con diverse condizioni operative. Tutti i reattori, comunque, hanno dato luogo a risultati instabili nel corso del processo di arricchimento del fango, spiegabili come conseguenza di fattori inibenti quali la presenza di ossigeno disciolto, concentrazioni elevate di nitriti e ammoniaca e l'indesiderata presenza e coesistenza di gruppi batterici che competono per gli stessi substrati. Una chiara evoluzione della composizione microbica è stata evidenziata dalla somiglianze tra le bande risultanti dalla DGGE condotta su campioni di fango con DNA amplificato, estratti in differenti istanti del processo di arricchimento dal reattore inoculato con fango attivo.

Le sostanze polimeriche extracellulari (EPS) svolgono un ruolo importante nel processo di aggregazione biologica delle biomasse ed influenzano il processo di arricchimento delle biomasse Anammox. Pertanto, l'evoluzione delle caratteristiche delle EPS è stato esaminato attraverso una serie di analisi qualitative e quantitative. Durante il processo di arricchimento delle biomasse Anammox è stato osservato un incremento nella produzione delle EPS e un decremento del valore del rapporto tra proteine e polisaccaridi (PN/PS). La matrice tridimensionale eccitazione-emissione (3D-EEM) ha mostrato una collocazione simile dei picchi di fluorescenza in tutti i campioni di EPS mentre in quelli estratti dalla biomassa Anammox ha mostrato due picchi distinti nel campo delle basse lunghezze d'onde di eccitazione. Il cromatogramma a raggi UV ha mostrato un incremento sia nell'intensità che nel numero di picchi, mentre i cromatogrammi a fluorescenza hanno mostrato un numero simile di picchi e solo un incremento nell'intensità. Un incremento dell'idrofobicità delle EPS è stato inoltre osservato durante il processo di arricchimento del fango.

Infine, il tema di questo lavoro di tesi si inserisce nell'ambito della gestione delle risorse microbiche (MRM) che si compone di un "input dato dall'ingegnere", "una scatola nera microbica" e un "output del processo". In questa tesi sono stati infatti condotti una serie di studi che riguardano l'"input dato dall'ingegnere", quali l'arricchimento della biomassa Anammox e la conduzione di un processo in reattore singolo di parziale nitrazione/Anammox, allo scopo di raggiungere i due principali "output del processo" rappresentati da un elevato rendimento di rimozione dell'azoto e da un rapido arricchimento della biomassa Anammox. Per esaminare le dinamiche della "scatola nera microbica" la tecnica molecolare di PCR-DGGE è stata condotta su campioni di biomassa prelevata in diversi istanti del periodo di

arricchimento. Analisi quantitative e qualitative sulle EPS sono state eseguite allo scopo di individuare la correlazione esistente tra le caratteristiche delle EPS e i parametri del processo di arricchimento.

Résumé (French)

Pour répondre aux exigences des limites de concentration en azote émises par les usines de traitement des eaux usées (STEU), l'élimination biologique de l'azote est devenue un processus courant grâce à son efficacité d'élimination. L'ajout des voies métaboliques nitrification / dénitrification dans les procédés classiques à boues activées est appliqué à grande échelle pour les eaux usées municipales et industrielles depuis les années 1960. Cependant ces dernières années, la découverte de l'oxydation anaérobie de l'ammonium (Anammox) offre une voie alternative pour éliminer l'ammonium des eaux usées. Dans ce procédé, l'ammonium est oxydé par voie anaérobie en azote gazeux avec les nitrites comme accepteur d'électrons, par des bactéries autotrophes. Les avantages - une demande en oxygène inférieure, aucune nécessité d'apport de source de carbone et une empreinte écologique plus faible - en ont fait une option attrayante pour le traitement biologique des eaux usées. L'objectif de ce travail est alors "d'étudier le processus d'enrichissement en Anammox ainsi que l'évolution de la diversité microbienne et les caractéristiques des EPS, pour le traitement des eaux usées dont le rapport C/N est faible".

En raison de son taux de croissance extrêmement lent et de sa rareté dans les boues enrichies, l'application à grande échelle du processus Anammox est entravée. Ainsi, le processus d'enrichissement de la biomasse Anammox à partir de trois types de boues d'ensemencement classique a été étudié: boues aérobie, boue de dénitrification et boues anaérobies floculantes. Les effets du contrôle de l'oxygène et de la pression de sélection ont été étudiés. Le processus Anammox a été mis en place avec succès dans le réacteur ensemencé avec les boues de dénitrification, avec une élimination de l'azote total d'environ 80% sous contrôle strict de l'oxygène, au bout de 150 jours. L'enrichissement a été confirmé par surveillance de la composition chimique de l'affluent / effluent et l'évolution de la diversité microbienne a été mis en évidence via les empreintes issues des électrophorèses sur gel dénaturant à gradient (DGGE) après réaction de polymérisation en chaîne (PCR). Avec un temps plus court de rétention hydraulique (HRT) et pas de contrôle strict de l'oxygène, le réacteur ensemencé avec les boues aérobies atteint une élimination totale de l'azote de 50-60%, après 240 jours. Les réacteurs ensemencés avec les boues anaérobies n'ont pas pu établir le processus Anammox, malgré deux essais avec contrôle de fonctionnement différent. Tous les réacteurs ont subi une fluctuation de leurs performances au cours du processus d'enrichissement, ce qui serait la

conséquence de facteurs inhibiteurs tels que l'oxygène dissous, l'absence de nitrites et d'ammoniac ainsi que la coexistence de bactéries indésirables qui sont en concurrence pour le même substrat. Une nette évolution de la composition microbienne a été démontrée par la similitude des bandes DGGE issues des échantillons d'ADN amplifiés et extraits à différentes étapes des réacteurs ensemencés avec différentes boues aérobies d'enrichissement.

Les substances polymériques extracellulaires (EPS) sont susceptibles de jouer un rôle important dans la bioaggrégation et ont une forte corrélation avec le processus d'enrichissement en Anammox. L'évolution des caractéristiques des EPS a été étudiée à travers une série d'analyses quantitatives et qualitatives. Une augmentation du rendement total d'extraction des EPS et une diminution du rapport protéine, polysaccharide (PN / PS) ont été observés au cours du processus d'enrichissement. Les matrices d'excitation émission à trois dimensions (3D-EEM) ont montré un pic de fluorescence localisé dans une zone similaire pour tous les échantillons d'EPS tandis que les échantillons avec les bactéries Anammox possèdent deux pics distincts dans le bas de gamme d'onde d'excitation. Pendant l'enrichissement, les empreintes issues de la chromatographie d'exclusion stérique couplée à une détection par absorbance en ultraviolet (UV) à montré une augmentation en intensité et en nombre de pics, alors que chromatogrammes issus de détection par fluorescence des PN-like ont montré un nombre de pics similaires avec seulement une hausse de leur intensité. Une augmentation de l'hydrophobicité des EPS a également été observée au cours du processus d'enrichissement.

Pour conclure, l'étude se positionne dans le cadre de la gestion des ressources microbiennes (GRM), qui est composée d' "ingénierie", d'une "boîte noire microbienne" et de "procédé". Dans cette thèse, une série d'études sur l''"ingénierie" : l'enrichissement en biomasse Anammox et le test du procédé nitritation partielle/Anammox en un seul réacteur, ont été menés, visant à aboutir à la GRM pour l' «élimination élevée de l'azote » et pour l' «enrichissement accéléré ». Pour révéler la dynamique de la «boîte noire microbienne", une analyse moléculaire par PCR-DGGE a été réalisée en fonction du temps au coursde la période d'enrichissement. Les analyses quantitative et qualitatives des EPS ont été menée en vue d'établir la corrélation entre les caractéristiques des EPS et des paramètres du procédé.

Chapter 1

Introduction

Chapter 1 Introduction

1.1 Source and sink of reactive nitrogen pollutant

1.1.1 Problem of reactive nitrogen

Element nitrogen (N) is number seven in the periodic table with oxidation state from -3 to +5. Both nitrogen compounds and nitrogen gas (N_2) are of crucial important in biosphere. N_2 gas is one of the inert gas which composes the largest proportion (78% volume) of air in the earth's atmosphere. Nitrogen is one of the major elements in life organisms which accounts for 10-15% of the total organic content. It is a central atom in proteins and nucleic acids. Under certain chemical or biological conditions, nitrogen under goes turnover between different oxidation states, which is known as nitrogen cycle (Fig. 1.1) (Hatfield and Follett 2008). The conversion of reactive nitrogen is mediated by a series of microorganisms, including ammonium oxidation bacteria (AOB), nitrite oxidation bacteria (NOB), Anammox and denitrifiers.

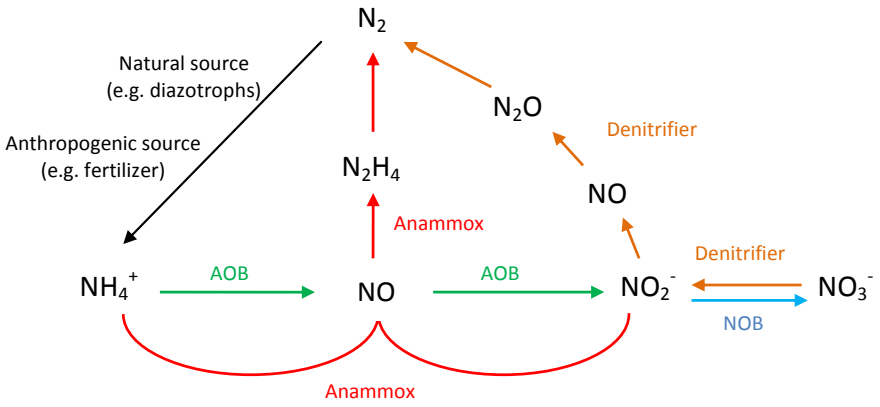

Fig. 1.1 Nitrogen cycle

Except the element nitrogen gas, nitrogen existing in other oxidation states are so called reactive nitrogen. Reactive nitrogen may pose threat to both human and ecosystem when it exceeds certain concentration. Thus is important to understand the conversion between reactive and non reactive nitrogen. Two major processes are responsible for the global nitrogen fixation from atmosphere. Firstly the biological nitrogen fixation is performed solely by highly diversed diazotrophic microorganisms which scattered in domain bacteria and archaea. Diazotrophs are featured by possessing the *nif* genes encoding for the enzyme nitrogenase, which is able to reduce N_2 to the reactive form, mostly ammonia (Zehr et al.

2003). They can be either free living (such as *Clostridium* in deep soil and *Desulfovibrio* in ocean sediments) or mostly plant associated symbiotic microorganisms (such as *Rhizobia* in *Fabaceae* plants and *Anabaena* in fern *Azolla*) (Izquierdo and Nuesslein 2015). Nitrogen fixation from diazotrophs accounts for 138 TgN/year which is 47.9% of the total global fixation (Fig. 1.2). Besides, lighting is another natural source of reactive nitrogen which amount to 5.4 TgN/year (Galloway et al. 2004; Galloway et al. 2008). Secondly, anthropogenic fixation of nitrogen became second most significant contribution in global nitrogen fixation since the discovery of the Haber-Bosch process in 1910s. The process involves the catalytic synthesis of ammonia from hydrogen and nitrogen gas under hyper condition (400 - 500˚C and 15-25 MPa) and metal catalyst. The application of the Haber-Bosch process secured the demand for fertilizer in agriculture to meet the need of increasing world population (Cherkasov et al. 2015). However it results in excess release of reactive nitrogen amount to 120 TgN/year (Galloway et al. 2008). Fossil fuel combustion is another major source of reactive nitrogen source but of less significance which equals 25 TgN/year (Fowler et al. 2013; Galloway et al. 2008). Fig. 1.2 shows that anthropogenic production of reactive nitrogen composes half of the total global production. The disturbance of the nitrogen balance eventually results in the accumulation of reactive nitrogen if they are not removed from the wastewater before discharging. Nitrogen in wastewater mostly exists in the form of ammonium (NH_4^+). A high concentration may harm the aquatic ecosystem in two aspects: (i) free ammonia (NH_3) is toxic (chronic) to microorganisms at concentration of 0.25 mgNH_3/L which is equilibrant to about 5-17 mg NH_4^+/L depending on the pH of the water (Brinkman et al. 2009); (ii) nitrification by microorganism require high amount of dissolved oxygen which leads to the hypoxia of the water body which leads to loss of fish and (iii) eutrophication leading to excessive algal growth (Erisman et al. 2013). Nitrogen also poses threat to human health when the drinking water is contaminated by high amount of nitrate. Nitrate, when reduced to nitrite, will cause an elevation of methemoglobin in red blood cell which contains ferric iron instead of ferrous iron thus has lower oxygen binding capacity and eventually cause tissue hypoxia. This is so called methemoglobinemia. Since infants are the most susceptible group to methemoglobinemia, it is also named as “blue baby syndrome” (Knobeloch et al. 2000). Therefore, removal of nitrogen is mandatory in wastewater treatment plants. The Urban Waste Water Treatment Directive was established by European Union aiming at regulating the treatment of wastewater from municipal and industrial sectors and serving as a baseline for each member state to form their own standard. According to the Commission Directive 98/15/EC, maximum concentration of discharged total nitrogen is 15

mgN/L for 10,000 – 100,000 population equivalents (P.E.) and 10 mgN/L for > 100,000 P.E. Physico-chemical and biological approaches have been employed to removal nitrogen over the past 100 years and new techniques are still being discovered and applied.

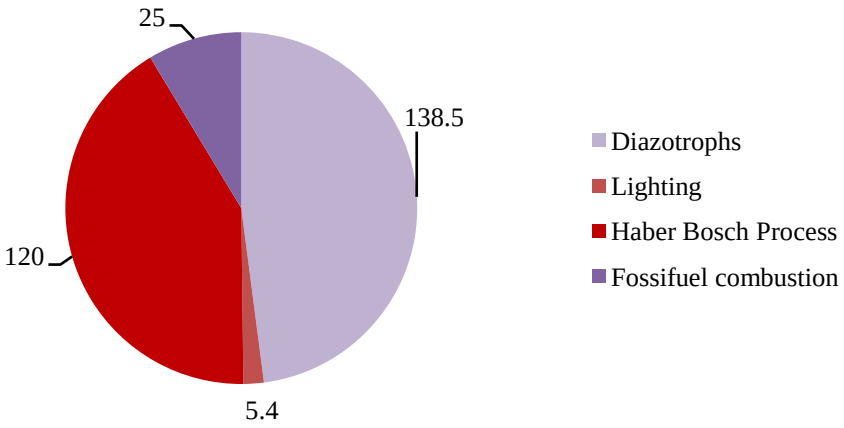

Fig. 1.2 Source of reactive nitrogen (TgN/year)

1.1.2 Physico-chemical removal of nitrogen

Stripping is the only physical removal of ammonium which separates the gaseous form of NH_3 from the water under alkaline condition. Both air and steam can be used as carrying bubble. In the case of air bubble, the resultant ammonia containing air is scrubbed by acid to form $(NH_4)_2SO_4$ or NH_4Cl solution which can be further concentrated and purified and subsequently used for fertilizer (Zhao et al. 2015). For steam stripping of ammonia, the continuous recirculation of the steam finally results in concentrated ammonia solution which is ready for further use (Zeng et al. 2006). The process has the advantages of low cost and simple operation. But also has disadvantages of low efficiency and sensitivity over low temperature (Capodaglio et al. 2015).

Breakpoint chlorination is a process which ammonium is oxidized to nitrogen gas by chlorine. Chlorine is added to ammonia containing solution until the residual chlorine reaches a minimum (breakpoint). During the process, both ammonium and other oxidizable pollutants are removed (Jeong et al. 2014). The major advantages of this process are the low capital cost, high efficiency and terminal oxidation of ammonia into non reactive nitrogen gas. However the drawbacks are: (i) incomplete oxidation of ammonium will produce chloramines which give disturbing odor to the water and (ii) in case organic matters are present, hazardous $CHCl_3$

could be formed which need further treatment. Furthermore, the high demand of chlorine is of environment and safety concern (Capodaglio et al. 2015).

Ion exchange process could be also used for ammonium removal. A natural zeolite clinoptilolite is known to be selective for ammonium relative to calcium, magnesium and sodium. In practice, wastewater passes through columns packed with 20*50 mesh sieved clinoptilolite, ammonium is absorbed by the clinoptilolite until maximum capacity of the zeolite is saturated. Regeneration of the column could be achieved through washing by caustic liquid or brine water (Almutairi and Weatherley 2015). The main advantages are the high efficiency and process stability. Whereas the disadvantages are the high capital and operational cost, complex process control and additional regeneration requirements (Capodaglio et al. 2015).

Struvite precipitation is another approach to simultaneously remove ammonium and phosphate in the wastewater with the addition of MgO under alkaline condition (pH 8.5-10). The formed struvite crystals ($MgNH_4PO_4.6H_2O$) could be used as fertilizer. Struvite precipitation is normally intended for phosphate removal. If ammonium removal is the main purpose, additional phosphate may be necessary when P/N ration is less than 1 (Latifian et al. 2014). Despite that the process simultaneously removes ammonium and phosphate, its high cost and complex operation hindered its wide application (Capodaglio et al. 2015).

Except the breakpoint chlorination, all the physical and chemical nitrogen removal processes are actually nitrogen recovery from wastewater into concentrated and stable form. The increasing fertilizer price makes chemical nitrogen recovery attractive. However over the past few decades, biological nitrogen removal became mainstream process for both domestic and some industrial wastewater treatment. As Mulder (2003) stated, biological nitrogen removal is the most cost efficient option for wastewaters containing up to 5 gN/L, which is applicable for most domestic and industrial wastewater.

1.1.3 Conventional biological removal of nitrogen

Since its discovery, activated sludge system has been widely implemented over the past hundred years and had significant contribution in the removal of reactive nitrogen from

wastewater. Activated sludge system is the most mature and widely applied wastewater treatment technology for the simultaneous removal of nitrogen and organic carbon (Metcalf et al. 2013). The process is based on the naturally occurring water purification process, which involves a group of microorganisms performing nitrification (Eq.1 and 2) and denitrification (Eq.3) (Metcalf et al. 2013).

$NH_3 + 1.5O_2 \rightarrow NO_2^- + H_2O + H^+$ (Eq.1)

$NO_2^- + 0.5O_2 \rightarrow NO_3^-$ (Eq.2)

$4NO_3^- + 5[CH_2O] + 4H^+ \rightarrow 2N_2 + 5CO_2 + 7H_2O$ (Eq.3)

Nitrification is accomplished by two groups namely ammonium oxidizing bacteria (AOB) and nitrite oxidation bacteria (NOB). Both are obligate aerobic and autotrophic bacteria which are able to work under dissolved oxygen (DO) level higher than 1 mgO_2/L. Nitrification process consumes considerable amount of alkalinity (described as $CaCO_3$) thus acidifying the water which could be used as an indicator of nitrogen contamination. Denitrification is a process where nitrate is reduced to nitrogen gas by denitrifiers (Eq. 3). Denitrifiers are heterotrophic and facultative anoxic bacteria working under anoxic condition using nitrate or nitrite as electron acceptor. Nitrification is normally combined with organic carbon oxidation by heterotrophic bacteria in activated sludge systems (Metcalf et al. 2013).

Different configurations of activated sludge systems have been developed and currently in practice, including the conventional completely stirred tank reactor (CSTR), plug flow and sequencing batch reactor (SBR), etc. Generally the activated sludge part of any wastewater treatment plant is composed of an aeration tank for carbon oxidation and nitrification, denitrification zone and settling tank to separate the effluent and sludge. Denitrification could be placed before the aeration tank (pre-denitrification) in order to make the influent organic carbon available to denitrifiers (Henze 2008). A system incorporating the phosphate removal by phosphate accumulating organisms (PAO) was developed for simultaneous removal of organic carbon, ammonium and phosphate (Kerrn-Jespersen and Henze 1993).

1.2 History of Anammox

1.2.1 Discovery

Before anaerobic ammonium oxidation (Anammox) was observed in 1992, denitrification was believed to be the only natural sink of reactive nitrogen. Anammox process is thermodynamically feasible according to the calculation of Gibbs energy from thermodynamic tables (Egli 2003). Ammonium removal at the expense of nitrate generation was firstly observed in a denitrifying pilot plant in Delft, the Netherland, in 1992 (Mulder et al. 1995). Since then the term "Anammox" was introduced and efforts on reproduction and purification of the specific bacteria have been conducted. By 1997, it was confirmed that the intermediates hydroxylamine and hydrazine were involved in the overall reaction based on N^{15} and N^{14} labeling on ammonium and nitrite from a 60% enriched mixed culture (vandeGraaf et al. 1997). However, the metabolic pathway was overwritten by Kartal et al (2011), who suggested that nitric oxide instead of hydroxylamine is the real precursor for the synthesis of hydrazine.

Later Strous et al. (1999b) found that sequencing batch reactor is good option for Anammox enrichment and achieved 70% enriched sludge. The overall reaction (Eq.5) was then proposed and later validated by most of the studies on Anammox (Strous et al. 1999b).

$$NH_4^+ + 1.5O_2 + 2HCO_3^- \rightarrow NO_2^- + 3H_2O + 2CO_2 \quad \text{(Eq.4)}$$

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- \rightarrow 1.02N_2 + 2.03H_2O + 0.066CH_2O_{1.5}N_{0.15} + 0.26NO_3^- \quad \text{(Eq.5)}$$

Again, Eq. 5 was rewritten by Lotti et al. (2014) through the use of high purity Anammox biomass cultivated in well controlled membrane bioreactor (MBR) (Eq.6).

$$NH_4^+ + 1.146NO_2^- + 0.071HCO_3^- + 0.057H^+ \rightarrow 0.986N_2 + 2.002H_2O + 0.071CH_{1.74}O_{0.31}N_{0.20} + 0.161NO_3^- \quad \text{(Eq.6)}$$

The use of gradient Percoll centrifugation yielded 99.6% pure culture which is able to perform 16s rRNA based genomic analysis. The organism identified was named *Candidatus Brocadia anammoxidans* (Strous et al. 1999a). Since then the research on Anammox process, inhibition and diversity became hot topic which is reflected by the exponential increasing in published articles (Zhang and Liu 2014). Several interesting facts about Anammox bacteria cells have been revealed. For example, the genome sequence of *Candidatus Kuenenia stuttartiensis* was

identified by Schmid et al. (2000); an organelle which is specific to Anammox bacteria was discovered and named "anammoxosome" (Lindsay et al. 2001; Schalk et al. 2000); ladderane was found to compose the anammoxosome membrane, which gave its rigid structure and lower permeability to the toxic hydrazine (Damste et al. 2002); an alternative metabolic pathway was discovered which Anammox bacteria dissimilatorilly oxidize organic carbon such as formate, acetate and propionate with nitrite or nitrate as electron acceptor (Kartal et al. 2007). The discovery of Anammox enabled a more efficient and economical wastewater treatment alternative for the removal of nitrogen. Anammox was also believed to contribute to 24-67% of total nitrogen sink in the continental shelf sediment (Thamdrup and Dalsgaard 2002) and 30-50% in the ocean (Devol 2003).

1.2.2 Molecular identification

The catabolism of Anammox was proposed to be via hydroxylamine (NH_2OH) and hydrazine (NH_2NH_2). The overall reaction is shown in Eq.5 ($\Delta G°' = -358$ kJ/mol NH_4^+). Anammox bacteria are exclusively monophyletic members of the phylum *Planctomycetes* (Strous et al. 1999a). *Planctomycetes* and their nearest relatives, the *Chlamydiae*, are the only known cell-wall containing bacteria that lack peptidoglycan. Instead, protein is major cell wall component in *Planctomycetes* (Fuerst 2005). Since no pure Anammox cultures are available so far which is due to its slow growth rate, the described species are all *"Candidatus"* species. The Anammox which are described so far, belong to the genera *Brocadia*, *Kuenenia*, *Scalindua*, *Anammoxoglobus* and *Jettenia*. In the marine environment, close relatives of *Scalindua* are found (Kuypers et al. 2003), whereas in most engineered systems, Anammox are related to *Brocadia* or *Kuenenia* (Ali and Okabe 2015).

1.2.3 From lab to full scale application

The conversion of ammonium to N_2 through Anammox pathway requires a partial nitritation step, in which about half of the ammonium is oxidized to nitrite (Eq.4). The two step autotrophic nitrogen removal process can be arranged both in two separate reactors system and one single reactor system. In a single reactor system, the nitritation and Anammox processes are separated by time (pulse DO control) and oxygen gradient (biofilm or granular) (Vlaeminck and Verstraete 2009). In two reactor system, partial nitritation is achieved by

SHARON process, the effluent of which will be treated separately by Anammox (van Dongen et al. 2001). A detailed comparison of the one reactor and two reactor systems has been given in a recently published review by Jaroszynski and Oleszkiewicz (2011). Taking into account the cost effectiveness and the concept of process intensification, the single reactor system has attracted intensive research interest and attempt in full scale application. Various single reactor systems have been developed by different authors resulting in a variety of removal rates. **Table 1.1** gives some examples of the single reactor system and their main features.

Table 1.1 Different single reactor systems

Name	Example of application	Removal rate	Reference
CANON	The process is operated in an SBR with periodic aeration, settling and effluent withdrawal. The nitritation takes place in the bulk while anammox bacteria grow in the anoxic core of the CANON granules.	350-910 mgN/L.d	Vazquez-Padin et al. (2010)
OLAND	A rotating biological contactor (RBC) with a contact surface of 1.32m^2 was used.	700 mgN/L.d	Vlaeminck et al. (2009)
DEMON	The process is characterized by a full scale sequencing batch reactor (SBR) with an intermittent aeration system controlled by the pH signal.	About 90%	Wett (2006)
MABR	A gas permeable carbon tube was covered with nonwoven fabrics to support biofilm. A counter-diffusion biofilm was used where nitrifying layer locates at the membrane-biofilm interface and anammox layer at the biofilm-bulk interface.	88.5%; 770 mg N/L.d	Terada et al. (2007); Gong et al. (2007)

Full scale application of the Anammox is still very much hindered due to its extremely slow growth with doubling time of 11-13 days as well as the scarcity of enriched seeding sludge. The first full scale Anammox based WWTP took about 2 years to start up (van der Star et al. 2007). By 2014, there are 96 full scale Anammox based WWTP installations in operation and 10 under construction worldwide (Ali and Okabe 2015).

1.3 Role of EPS

EPS are sticky fluids excreted by cells especially when under stress. The production of EPS is essential in the formation of floc, aggregates and biofilm (Liu et al. 2004). The roles of EPS have been believed to include: structural formation and maintenance of aggregates or biofilm; increase of the substrate diffusivity; influence aggregate morphology by EPS hydrophobicity;

the composition of EPS might influence biofilm or granule formation (Sheng et al. 2010). The composition of EPS mainly includes not only polysaccharides, proteins, lipids, nucleic acids, uronic acides and inorganic components (Sheng et al. 2010) but also more complex macromolecules such as glycoproteins or proteoglycanes (Bourven et al. 2014). Different studies on EPS in granular formation have been conducted for the granulation process of aerobic and anaerobic sludge in which the protein:polysaccharide (PN:PS) ratio has been used as an indicator (Ma et al. 2012).

Study of EPS from activated sludge system has been intensively conducted aiming at improving the settling and flocculation ability (Bala Subramanian et al. 2010; Wilén et al. 2003b). It was found that different composition of EPS has different and complex impact on the total sludge flocculation. For example, flocculation of sludge increases with increase in proteins and decrease in humic-like substances (Wilén et al. 2003a). It was also believed that the negatively charged EPS has negative impact on the sludge aggregation and settling due to the increased repulsive force between cells (Sheng et al. 2010). The classic Derjaguin, Landau, Vervey, and Overbeek (DLVO) theory offers a way for further study of sludge aggregation in relation to total EPS and each of its composition. However, the lack of standard methods in the EPS extraction and analysis rendered the comparison of different research difficult (Liu et al. 2004). Sheng et al. (2010) summarized methods for EPS extraction and analytical techniques used in EPS research in a recently published review. In general, physical extraction methods have lower efficiency than the chemical methods. However chemical methods have problems of cell lysis and/or contamination which disturb further analysis. Cation exchange resin method (CER) has gradually become the mainstream extraction method for EPS research because of its high efficiency, low cell lysis and no contamination (Frolund et al. 1996; Nielsen et al. 1996). A detailed literature review on EPS is presented in Chapter 2.

1.4 Problem statements

Due to the low growth rate and poor cultivability of Anammox bacteria with a generally acknowledged doubling time of 11 - 13 days, the long start up time has been a major bottleneck of its application (Strous et al. 1999b). In order to facilitate a “fast” start-up, various configurations have been tested in forming aggregates and biofilms, including SBR, Membrane Bioreactor (MBR), Upflow Anaerobic Sludge Blanket (UASB) reactor, Moving

Bed Bioreactor (MBBR), Upflow Biofilm Reactor (UBF), etc (Chen et al. 2010; Tao et al. 2012; Zekker et al. 2012). The time needed for occurrence Anammox activity was observed mostly around 2-4 months and a stable nitrogen removal rate was reached more than one year after cultivation started. This complies well with a modelling results conducted by Dapena-Mora et al. (2004) using the package WEST. As an alternative, preservation and reactivation of Anammox biomass as a method to quick start-up has also been reported to be feasible (Vlaeminck et al. 2007). The enrichment of Anammox experiences three phases as indicated by NH_4^+, NO_2^- and NO_3^- concentrations (Chamchoi and Nitisoravut 2007; Wang et al. 2011). In the first phase which lasts about 3-5 weeks, the culture experience endogenous denitrification with no Anammox activity, in which the effluent ammonium concentration is higher than the influent due to the cell lysis and break down of organic nitrogen. The second phase involves the shift and enhancement of Anammox composition due to artificially induced substrate constraint. Due to the low growth of Anammox, this phase can last as long as more than 10 weeks. The last phase allows the stable and optimum nitrogen removal from Anammox activity.

The **importance of inocula source** has been reported towards the conclusion that inoculums with high concentrations of Anammox bacteria show better performance in enrichment especially in terms of shorter enrichment period and higher removal efficiency after steady state is reached (Wang et al. 2011). However, Jeanningros et al. (2010) concluded that "deammonification inoculum does not play an important role" in a pilot scale deammonification plant. The impact of the initial inocula and the criterion of the selection have not been fully understood in literature and will be investigated.

One-reactor systems often face the problems of difficulties in oxygen control in which the DO should be able to cope with varying influent ammonium concentration and the available oxygen should be just enough to partially oxidize the ammonium to nitrite. Also the existing nitrite oxidizing bacteria (NOB) may compete with Anammox for inorganic carbon and nitrite.

1.5 Objectives of the thesis

The main objective of the study is "*to investigate the process of Anammox enrichment and evolution of microbial diversity and EPS characteristics for treating wastewater with low C/N ratio*".

The specific objectives are:

- To study the effect of seeding sludge and operation strategies on the enrichment of Anammox biomass (Chapter 3);
- To investigate the evolution of microbial diversity during the enrichment process (Chapter 3);
- To investigate the evolution of quantitative and qualitative EPS characteristics during the enrichment process (Chapter4);
- To compare the EPS characteristics between reactors with different process performance (Chapter 4);
- To conduct a preliminary study on the feasibility of one-stage partial nitritation/Anammox sequencing batch reactor with minimum operation control (Chapter 5).

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Chapter 2

Role of extracellular polymer substances (EPS) production in bioaggregation: application to wastewater treatment

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Chapter 2 Role of extracellular polymer substances (EPS) production in bioaggregation: application to wastewater treatment

Summary

This paper reviews the formation, structure and stability of bioaggregates with an emphasis on the composition and distribution of extracellular polymeric substances (EPS) and their role in bioaggregation. Bioaggregation is ubiquitous in natural environment and is of great importance in biological wastewater treatment processes. It greatly influences the flocculability, settleability and dewaterability for flocs and sludge retention and shear resistance for biofilms. The physico-chemical and microbial structures of bioaggregates are dependent on operational conditions as well as microbial diversity and spatial distribution. The formation of bioaggregates is mediated by the physico-chemical interactions as well as the microbial interactions such as EPS production and quorum sensing.EPS are composed of a mixture of macromolecules including proteins, polysaccharides, humic-like substances and nucleic acids, which entrap the microbial cells in a three dimensional matrix. The composition and physico-chemical characteristics of EPS have significant influence on the maintenance of the bioaggregates structure and the process performance of the wastewater treatment. However, the mechanisms of bioaggregation are still unclear and the conclusions on the role of EPS were mostly drawn from the established correlations and hypotheses. This paper expects to provide up-to-date knowledge on bioaggregation and insights for further studies and applications.

Keywords: EPS, granulation, flocculation, mechanism, protein, polysaccharide

2.1 Introduction

Engineered biological wastewater treatment processes have been extensively applied in treating all kinds of wastewaters, such as municipal wastewater and industrial wastewater. The engineered processes are enhanced from the naturally occurring microbial processes through controlled operational techniques, which could achieve much higher biomass concentration and enhanced removal of soluble biodegradable pollutants. Some examples include the activated sludge system, up-flow anaerobic sludge blanket (UASB) system and partial nitritation/anaerobic ammonium oxidation (Anammox) system (Metcalf et al. 2010; Mulder et al. 1995). Activated sludge systems have been widely implemented as a mainstream process for municipal wastewater treatment, in which organic carbon is aerobically consumed and removed by heterotrophic bacteria, and in some cases nitrogen is simultaneously removed by co-existed microorganisms through nitrification-denitrification pathway (Metcalf et al. 2010). Microorganisms are mostly present in the form of flocs in activated sludge system (von Sperling 2007). In UASB systems, the microorganisms exist in the form of granules performing anaerobic digestion for organic carbon removal. UASB systems are sometimes used as pretreatment for activated sludge process to reduce the strength of organic carbon load (Zhang et al. 2013). It could also be implemented as mainstream process treating high strength wastewater rich in organic carbon such as pulp and paper wastewater, food processing wastewater and waste activated sludge (Kalyuzhnyi et al. 1998; Pradhan et al. 2015). Anammox process was discovered twenty years ago (Mulder et al. 1995), in which ammonium is anaerobically oxidized into nitrogen using nitrite as electron acceptor in the absence of oxygen. The group of microorganisms which perform partial nitritation and Anammox tend to aggregate into biofilms in order to maximize the sludge retention (Wang et al. 2014b). The process has been attempted in full-scale application but still very limited cases could be found mainly due to the extremely slow Anammox bacterial growth (Ding et al. 2015; Ni and Zhang 2013).

Microorganisms in reactors have the ability to form concentrated bioaggregates and achieve higher treatment efficiency. Various types of bioaggregates could be found in lab scale or full-scale wastewater treatment systems. According to their physical structure, bioaggregates are categorized into flocs and biofilms. Biofilms are further divided into granules and attached growth systems. The physical status of the carrier further sub-categorizes the attached growth systems into static solid surface system (e.g. biofilter) and mobile solidsurface system (e.g. moving bed bioreactor). Some authors consider the attached

growth system as “biofilm” to differentiate flocs and granules (Karadag et al. 2015; Langer et al. 2014). To avoid confusion, the term “biofilm” includes granules and attached growth systems in this paper, which is in compliance with Nicolella et al. (2000) and Maksimova (2014).

The advantages of bioaggregation are: (i) to facilitate the solid-liquid separation, (ii) to enhance the sludge retention and (iii) to optimize the accessibility to nutrients as well as sytrophic association by the juxta-position of microorganisms (Sheng et al. 2010). However, there are also problems which may hinder the expected improvement in wastewater treatment process. One of the most frequent problems in activated sludge systems is the sludge bulking resulted from deflocculation, which hinders the downstream settling and dewatering process. Factors that may be responsible for the deflocculation of activated sludge include physical conditions such as temperature, shear stress and over aeration (Feng et al. 2009; Morgan-Sagastume and Allen 2005), chemical conditions such as the presence of chlorine, sulfide, heavy metals and anaerobic condition (Mascarenhas et al. 2004; Nielsen and Keiding 1998; Wilen et al. 2010) as well as microbial activity such as filament formation due to overgrowth of denitrifiers (Guo et al. 2014; Wilen et al. 2000). Compared to flocs, granules are compact and dense spherical bioaggregates that could be formed in both anaerobic and aerobic bioprocesses. The rigid structure of the granules gives them the advantage of higher resistance to shear stress. The compact and dense internal structure may provide protective role against toxic compounds through resistant in mass transfer (Liu et al. 2004b). However, the main drawback of the granular system is the long start-up time needed for the granulation process, which was reported as 2-8 months for anaerobic sludge and approximately 4 weeks for aerobic sludge (Liu et al. 2004b). Also aerobic granules were found to be of low stability with “no apparent cause” (Liu et al. 2004b). With respect to attached growth system, the formation of biofilm on carrier may play similar roles as granules but face another problem of biofilm detachment from its carrier. Furthermore, attached growth biofilm is undesirable when it causes membrane fouling problems (Boelee et al. 2014; Ferrera et al. 2015).

The formation of bioaggregates has been believed to be mediated and/or enhanced by EPS (Sheng et al. 2010). According to Tian et al. (2006), EPS constitute 80% of the mass of activated sludge. Hence the focus on EPS in studying the mechanism of microbial aggregation is important in formulating the strategies to overcome the above-mentioned problems. EPS are sticky materials which form a 3-dimentional matrix bound to the surface of the cells, resulting from cell secretion and cell lysis as well as molecules absorbed from the bulk solution (Wingender et al. 1999). The existence of EPS surrounding cells was confirmed by

microscopic analysis such as confocal laser scanning microscopy (CLSM) (de Beer et al. 1996). From the microbial point of view, EPS production is an expensive cell activity because the synthesis of these organic materials requires energy and reducing equivalents for both autotrophs and heterotrophs. This consideration makes interesting the benefit of EPS production which could balance the investment. Besides facilitating the aggregation of cells, some other functions which microorganisms may benefit include retention of water, sorption of exogenous nutrients, and providing a protective layer against harmful environment such as biocides, heavy metals etc. (Wingender et al. 1999).

Despite that bioaggregations are widely occurring in wastewater treatment biosystems, the mechanisms behind it are still unclear and most of the postulated models are based on hypothesis. On the other hand, activated sludge flocs have been intensively studied and applied for the past hundred years in contrast to anaerobic granules that have been investigated since 1980s and aerobic granules that have just got attention since late 1990s (Adav et al. 2008b; Mishima and Nakamura 1991; Peng et al. 1999). This paper aims at providing an up-to-date review on the structures, formation and stability of bioaggregates with a special emphasis on the composition and distribution of EPS and their role in the bioaggregation process. The scope of attached growth systems is not fully covered as a limitation of this mini-review mainly because this review focuses on cell to cell interaction while cell to carrier interaction is another large topic which is of crucial importance in attached growth system but out of scope in the present paper.

2.2 Characteristics of bioaggregates and EPS

2.2.1 Physico-chemical structure of bioaggregates

The physical characteristics of different types of bioaggregates determine the different approaches in studying its structure. Flocs were studied in bulk because the scale of its fractal structure is changeable in response to parameters such as shear stress and nutrients loading. Granules, as compact individual units which are independent of each other, display high resistance to variation of operational parameters. In attached growth systems, microorganisms present in bulk on the surface of the carriers while the inner structure of the bulk resembles granules. In general, two models have been proposed for all kinds of bioaggregates, namely layered model and fractal model. Based on that, several approaches including rheological

study, fractal characterization, direct microscopic visualization and mathematical modeling have been applied to study the structure. However, the extent of applicability and efficiency of these approaches differs among the different types of bioaggregates. The section herein discusses separately the structures of each type of bioaggregates.

Flocs

Two approaches have been applied to study the structure of flocs, the rheological and fractal approach. In the rheological approach, aggregates were hypothesized to be stratified into multiple layers. Mezger (2006) proposed a two-plate model in which the upper plate was readily removed by shear force and the lower plate was immobilized. Similarly, Liao et al. (2002) believed that the inner part of the floc was mainly linked by van der Waals and/or hydrophobic interactions while the outer layer was mainly governed by electrostatic, ionic interactions and hydrogen bonds which were closely related to sludge retention time (SRT). Sheng et al. (2006) formulated a multi-layer structural model in which a stable part was supposed to comprise the core while the dispersible part was located in the outer region. The dispersible part was further layered into loose part and compact part. In all of these layered models, EPS was hypothesized to act as gel matrix to hold cells together. The gel strength of EPS was proportional to the resistance of the floc to shear intensity. The gel-like EPS was believed to contribute to the non-*Newtonian* property of sludge mixed liquor (Legrand et al. 1998). Yuan et al. (2014) conducted a rheological study on fractionated EPS separated by different shear intensity. The results obtained by comparison of critical storage modulus of EPS and that of sludge after EPS extraction showed that tightly bound EPS (TB-EPS) might be the key fraction of the gel components of EPS. This multi-layer model was sufficiently applied in studying the floc structure with implications to flocculability, settleability and dewaterability of flocs (Huang et al. 2012; Li and Yang 2007; Yu et al. 2008).

Fractal characterization is a more detailed approach to study the floc structure. In this approach, the most important parameter is the mass fractal dimension d_F. According to the definition given by Gregory (1997), when the logarithm of mass is plotted against the logarithm of size (e.g. diameter, length), the slop of the linear plot is defined as d_F. An ideal compact homogeneous particle has a d_F value of 3, but for flocs it is normally less than 3. The value of d_F describes the compactness or looseness of aggregates. Snidaro et al. (1997) visualized the fractal structure of activated sludge flocs by transmission electron microscopy (TEM). It was observed that the predominant microflocs of 125 μm were composed of aggregates of 13 μm and those 13 μm aggregates were composed of 2.5 μm subunits. A d_F of

3 for the 13 μm unit was obtained by CLSM, which implied that the 13 μm units were the fundamental units of the fractal flocs. Gregory (1997) proposed a similar 2-dimensional model of "self-similar" floc structure under ideal assumptions. In this model, a triplet of equal spheres formed the fundamental unit and amplifications of this structure mimicked the flocculation process (Fig. 2.1). Recently Yuan et al. (2014) proposed a more realistic fractal model of floc (Fig. 2.2) which is linked to the classic Derjaguin, Landau, Verwey and Overbeek (DLVO) theory. In this study, TB-EPS as well as flocs after loosely bound EPS (LB-EPS) extraction had the highest d_F, hence TB-EPS was believed to be the gel reagent of the basic unit.

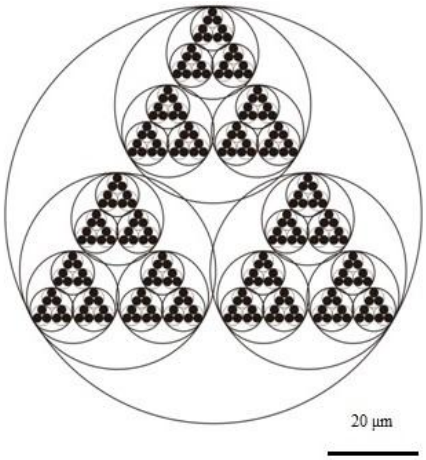

Fig. 2.1 Model of ideal floc fractal structure derived and adapted from Gregory (1997)

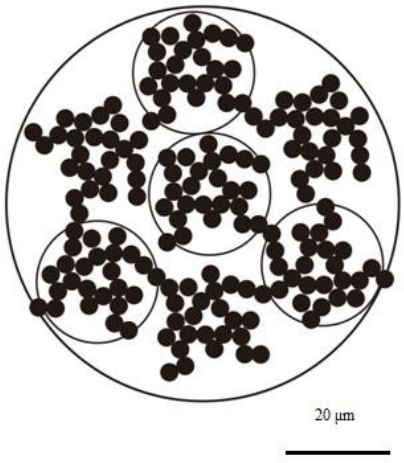

Fig. 2.2 Model of floc fractal structure under actual scenario derived and adapted from Yuan et al. (2014)

Granules

Unlike flocs, granules exhibit more compact and spherical structure and remain "independent" of each other. This enables that the studies of granules vary from micro-scale, which focus on the internal structure of an individual granule (Liu et al. 2010b), to macro-scale which focus on the operational strategies aiming at stimulating the granular formation (Wang et al. 2006) as well as its process performance (Ab Halim et al. 2015). Novel microscopic, staining and fluorescence techniques facilitate the full visualization of the internal structure of granules. Enzymatic staining also enables the visualization of EPS distribution over the whole granule (Miksch and Konczak 2012). Mathematical modeling is another method to predict and validate the behavior of granules at both micro and macro scale (Ni and Yu 2010).

Anaerobic granules have been intensively studied over the last 30 years while the research on aerobic granules is relatively new. Liu et al. (2003) reviewed the proposed structural model for anaerobic granules from UASB reactor, including *Cape Town's model*, *Spaghetti model*, *Syntrophicmicrocolony model* and *Multilayer model*. The authors concluded that no individual model could reasonably explain the whole granulation process and proposed a four step granulation process which is discussed in Chapter "Mechanisms of bioaggregates formation". Similarly, Hulshoff Pol et al. (2004) proposed that physico-chemical approach served as framework where biotic factors could be supplemented to form a unified granulation theory. Major differences of aerobic and anaerobic granules have been identified as: (i) longer cultivation time for anaerobic granules and (ii) lower stability for aerobic granules (Liu et al. 2009; Liu et al. 2004b). None of these relate to the internal physico-chemical structural and no literature, to the best of the authors' knowledge, claims the existence of a clear distinction in structure between anaerobic and aerobic granules although different microbial species may play key roles in granulation (Hulshoff Pol et al. 2004). Thus the discussion in this section does not discriminate these two granule types.

A *"sliced" granule model* (Fig. 2.3) was well developed and calibrated by Su and Yu (2006a) and Su and Yu (2006b), in which aerobic granules were assumed to be ideally spherical and the mass transfer of substrate and oxygen follows *Fick*'s Law of diffusion and the conversion of substrate by microorganisms follows Monod's kinetic equation. This model has been validated by data from different authors thus being useful in predicting the treatment performance of aerobic granular system in terms of substrate removal (Su and Yu 2006b). However, the sliced model did not consider the permeability and porosity of the granules, which have significant impact on the settling velocity as well as mass transfer within granules (Mu et al. 2008). Johnson et al. (1996) found that fractal aggregates settled 4-8.3 times faster than the predicted value calculated from *Stoke*'s Law. This observation was based on building

a *single particle fractal model* and verified by settling experiments on latex microspheres. However, this model might be over simplified for granules composed of microorganisms because non-bio particles were selected for the model verification. Microbial activities such as bacterial growth/decay, substrate conversion and EPS secretion were not taken into consideration. Thus the single particle fractal model might serve as a starting step in further revealing the granular structure. A *cluster fractal model* of microbial granules was developed by Mu et al. (2008), in which drag coefficient was found to be lower than that predicted by *Stoke*'s Law due to the porosity and permeability. The most important feature which differentiated the cluster model from the single particle modelwas that the primary particles within the granule were not uniformly distributed but separated into fractal principle clusters formed through coagulation. This "cluster model" was visualized by Gonzalez-Gil and Holliger (2014) who presented the fluorescence image of aerobic granules for nitrogen and phosphorous (N/P) removal stained by hematoxylin and eosin.

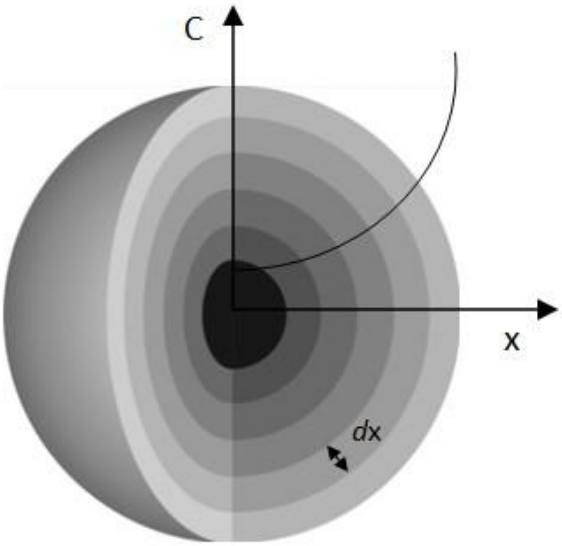

Fig. 2.3 Sliced granule model derived from Su and Yu (2006a); Su and Yu (2006c)

Granules were found to be highly porous with a porosity of 0.68-0.93 for aerobic granules and 0.64-0.90 for anaerobic granules (Liu et al. 2009; Mu et al. 2006). The porous feature of granules was reported to be caused by the inter-space of principle clusters as well as the decay of bacteria which left cavities inside the granules (Gonzalez-Gil and Holliger 2014). Li et al. (2011) observed that in aerobic granules, microorganisms were located on the surface and wall of channels; carbohydrates mainly were distributed on the surface and proteins were concentrated in the interior part. Inside pores could presumably facilitate internal mass transfer of substrate through convective flow and capillarity flow. Thus the mass transfer inside the granules may be mediated by a combination of *Fickian* flow, convective flow and

capillary flow. However, to the best of the authors' knowledge, no work has been conducted on granular models which considered the combination of all the three flows. Although granules are highly porous, the effective porosity could be much lower due to the pores dead end as well as pores clogging by EPS and cell debris (Lemaire et al. 2008). Also since EPS locate outside cell wall, it may cause resistance of substrate diffusion into the cell thus decreasing permeability.

Attached growth system

Attached growth systems were also found as porous structure with microorganisms entrapped in EPS matrix (Liang et al. 2013). However, as different from granules, carrier type and texture have significant impact on the biofilm structure in that different carriers may have different affinity to the colonized microorganisms (Felfoeldi et al. 2015). The affinity between carrier and colonized cells also contributes to the structure (Almstrand et al. 2014). Furthermore, the surface roughness is an important parameter for the performance of attached growth biofilm system while it is not the case for granular system (Jiang et al. 2015). Both clustered (Mahendran et al. 2012) and layered (Ning et al. 2014) biofilm structures have been reported in literature.

The consideration of physico-chemical structure provides a picture of general morphology of the bioaggregates and facilitates the understanding of the differences between flocs and biofilms. Flocs experience more aggregation-disintegration under the shear range as well as the variation in influent composition of the treatment process. Whereas biofilms are much more resistant to shear and environment change and remain "intact" during the treatment process. Also the performances of the wastewater treatment system, including settling and dewatering, could be well explained by the parameters such as density, permeability and porosity. However, in this approach the microorganisms and EPS were assumed to be "static" thus microbial activity was not taken into consideration. To fully understand the biological wastewater treatment process and take advantage of the benefit of bioaggregation, it is necessary to investigate in the microbial composition and its distribution within the bioaggregates and their role in the bioaggregation process.

2.2.2 Microbial spatial distribution inbioaggregates

Molecular biological techniques allow the visualization and quantification of microbial diversity and distribution inside the bioaggregates. Such methods should bring information about the microbial population playing active roles in the treatment process as well as the biological background of the aggregation mechanism. The section herein discusses about the microbial diversity and distribution of each type of bioaggregates at both steady state and during the granulation process.

Flocs

Flocs from activated sludge treating both industrial and municipal wastewaters have been most frequently studied. Due to its similar nutrient removal activity (organic carbon and nitrogen removal), it is not surprising that the dominating groups found in different wastewater treatment plants (WWTPs) are similar at least at phylum level. Among all the groups, *Beta-proteobacteria*, *Alpha-proteobacteria*, *Gamma-proteobacteria*and *Delta-proteobacteria*are presented in dominant numbers while *Plantomycetes*, *Firmicutes*, *Bacteriodetes* and *Cytophaga* were found in relatively lower numbers (Wilen et al. 2008). Some bacteria were found to be able to form flocs or compact microcolonies. For example, *Beta-proteobacteria* are mostly acknowledged as floc-forming and microcolony-forming bacteria which are often present in large microcolonies with diameter of 10-100 μm. *Alpha-proteobacteria* mostly grow as single cells or smaller microcolonies with diameter of 5-20 μm (Klausen et al. 2004). The distribution of the cells and microcolonies was found to be highly dependent on the microenvironment inside the flocs, specifically the distribution of nutrients and oxygen (Han et al. 2012). It was found that the distribution of nutrients and oxygen varied among different size of flocs. The microelectrodes analysis showed a steep concentration gradient of dissolved oxygen (DO), NH_4^+ and NO_3^- from the surface to the core of the flocs with a size of 100 μm. In flocs with size between 60-100 μm, the distribution of these chemical compounds was relatively even. In smaller sized flocs the concentrations of the chemical compounds were penetrated. The distribution of the chemical compounds was in good correspondence with the distribution of functional group of ammonium oxidation bacteria (AOB) and nitrite oxidizing bacteria (NOB), which can either be layered or evenly distributed (Han et al. 2012). All the cells and microcolonies were organized and entrapped in an EPS matrix, in which organic matters can be hydrolysed by extracellular enzyme (Yu et al. 2007; Zhang et al. 2015b). If only the morphology of the cells are considered independently of the cell species, the physico-chemical structure of flocs is fractal as described by Gregory (1997) and Yuan et al. (2014). However, if the groups and function of the microorganisms are

discriminated, the microbial community distribution within the flocs is heterogeneously organized according to the microenvironment. The mechanisms of the floc formation and EPS production is discussed in Chapter "Mechanisms of bioaggregates formation".

Granules

Thanks to the microscopic and molecular biotechniques, microbial distributions in granules were found to be arranged as homogeneous (Ni et al. 2010), clustered (Gonzalez-Gil and Holliger 2014) and concentric layered structure (Ivanov et al. 2008), which correspond to the respective single particle fractal, clustered fractal and layered physico-chemical structure. According to Gonzalez-Gil et al. (2001), such granule morphology variations were the results of microbial response to specific environmental conditions (i.e. substrate concentration gradient, feast-famine condition and predation).

Homogeneous structures were mostly observed in granules with low microbial diversity and performing single microbial process. Ni et al. (2010) studied the granules from a lab-scale Anammox UASB reactor and found that the dominant coccoid shaped cells, which was 95% similar to *Candidatus Brocadia anammoxidans*, evenly distributed over the granules observed by fluorescence in situ hybridization (FISH) and CLSM. Similarly, Gonzalez-Gil et al. (2015) found *Candidatus Brocadia* resembled Anammox microcolonies embedded in high and low dense EPS matrix in the granules from the Anammox reactor of a full-scale two stage partial nitritation/Anammox reactor. Other than the Anammox bacteria, the phosphorous removal "*Candidatus Accumulibacter phosphatis*" microcolonies (97.5%) were also found to be homogeneously distributed in aerobic granules from a lab-scale sequencing batch reactor (SBR) performing enhanced biological phosphorous removal (EBPR) process (Barr et al. 2010). Although most of the homogeneous distribution structures were found in single species or group granules, randomly distributed multicultural microcolonies in anaerobic granules was observed in earlier years (Dolfing et al. 1985).

Clustered structure granules were found with different microbial community composition (12.3% *Candidatus Accumulibacter phosphatis*, 57.9% *Candidatus Competibacter phosphatis*) in the same reactor of Barr et al. (2010). Another example of clustered EBPR granules was given by Gonzalez-Gil and Holliger (2014) who proposed that the clustered mature granule dominated by *Accumulibacter* was the result of outgrowth of bacteria rooting from the interior of the granules. Similar *Accumulibacter* and *Competibater* dominated clustered structure granules were cultivated by Weissbrodt et al. (2013) through different mechanisms. The abundant literature on the clustered structure from those two groups may imply the tendency

of the specific physiological strategy which might be genetically rooted. However, in fact the first clustered structure granule was found in a full-scale Expanded Granular Sludge Bed (EGSB) reactor treating brewery wastewater (Gonzalez-Gil et al. 2001). In this studied anaerobic granule, the methanogen *Methanosaeta spp.* was exclusively arranged in "white cluster" while the rest syntrophic eubacteria and hydrogenotrophic methanogens were embedded in "black" metal concentrated EPS matrix, presumably to facilitate "syntrophic association".

Layered structure granules are overwhelmingly found in the literature in both aerobic and anaerobic systems. The initial consideration of granule structure is believed to be concentric layered and the mass transfer is governed by *Fick's* Law. The hypothesis was well modelled and verified in different biofilm systems (Rittmann and Manem 1992; Su and Yu 2006a; Su and Yu 2006b). Layered arrangements of mixed culture granules have been continuously observed through microscopic techniques in both aerobic and anaerobic systems. Ivanov et al. (2008) found that in a 1 mm aerobic granule performing ethanol and nitrogen removal, AOB located from the surface till a depth of 0.55 mm and consisted of 69% of the total cell; enterobacteria located at 0.55-0.85 mm while *Bacteroides spp.* formed the anaerobic core. Similarly, Tay et al. (2002) found a three-layer structure of glucose fed aerobic granule, which was composed of the AOB *Nitrosomonas spp.* dominated layer on the edge, followed by an anaerobic layer dominated by *Bacteroides spp.* and another layer of dead cells towards the core. Recently, Lv et al. (2014b) observed similar structure of aerobic granules but was composed of different microbial families. In an acetate and propionate fed aerobic granule, the surface slice was found principally dominated by the fast growing obligate AOB *Micobacteriaceae* among other families while the core was mainly composed of the facultative anaerobic denitrifier family *Rhodocyclaceae* genus *Thauera*, which was also able to produce excess EPS. Zheng et al. (2006) studied the formation of glucose fed anaerobic granules by DNA probes and found that mature granules consist of *Methanosaeta concilii* in the core with syntrophic consortia adjacent to it and filamentous bacteria in the surface layer. Layered structure was found to be typical for coexisted partial nitritation/Anammox granules in which the Anammox bacteria with majority *Candidatus Brocadia* and *Candidatus Kuenenia* formed the core, and AOB with majority genus *Nitrosopira* and *Nitrosomonas* formed the rim (Vazquez-Padin et al. 2010; Vlaeminck et al. 2010; Volcke et al. 2010).

Attached growth system

Similar layered structures were also found in an attached growth system moving bed bioreactor (MBBR) consisting of AOB *Nitrosomonas* and Anammox *Candidatus Brocadia* (Almstrand et al. 2014). Clustered attached growth system was found by Mahendran et al. (2012) in which AOB and NOB present as micro-clusters attach to carrier media in an integrated fixed film activated sludge system.

2.2.3 Process dependent EPS composition and distribution

EPS composition

Production of EPS is a microbial activity of microorganisms triggered by environmental stress. Genes encoding EPS production have been identified in some strains from the wastewater treatment process. For example, genes assigning alginate production from phylum *Bacteroidetes* from an EBPR were characterized by Albertsen et al. (2013). Wan et al. (2015) also found that Psl and Alg genes expressed for exopolysaccharide production could enhance aerobic granulation induced by calcium precipitation as granule core. However, species specific investigations of EPS production are rare in literature. Badireddy et al. (2010) found that EPS produced by different microorganisms might be homologous in major components but different in minor ones such as lipids and phosphodiesters. Furthermore, in the context of wastewater treatment process, microorganisms exist as mixed culture and none of the EPS extraction methods could differentiate the EPS produced by specific microorganisms. Thus the discussion on EPS composition and distribution is not expected to be species specific but rather differentiated by function. The key components of EPS have been believed to include proteins, polysaccharides, humic-like substances, uronic acids and nucleic acids (Wingender et al. 1999). EPS was considered as gel material in the formation of bioaggregates through a cross-linked polymer matrix during cultivation (Seviour et al. 2009b). Comparisons of EPS global patterns from different sludge and substrate sources were attempted in different review papers (Liu et al. 2004b; Sheng et al. 2010). However, the results were always contradictory and a definitive conclusion yet to be drawn. Table 2.1 presents a few examples of EPS composition in different systems. Similar summary of earlier studies could be found in Liu and Fang (2003). Besides the global composition and concentration of EPS, more detailed studies on specific EPS composition were conducted. For example, alginate-like polysaccharide (Lin et al. 2010) and granulan (Seviour et al. 2011) have been elucidated through molecular techniques, such as Fourier Transform-Infrared spectroscopy (FT-IR) and

Gas chromatography–mass spectrometry (GC-MS). They were considered and believed to play gelation role in biogranulation. Proteomic technique provides informative approach for extracellular protein characterization. For example, Zhang et al. (2015b) investigated the extracelluar protein composition of anaerobic, anoxic and aerobic sludge from different tanks of the same WWTP. *Proteobacteria*, *Firmicutes* and *Bacteroidetes* were found to be the dominant groups in all the sludge samples. The main role of the extracelluar protein was found to be multivalent cation binding, which was similar among different sludges, and catalytic activity (i.e. extracelluar enzyme), among which anaerobic sludge showed more variety especially abundant in hydrolase. Furthermore, the physico-chemical properties of protein secondary structure were found to affect aggregation process in activated sludge (Badireddy et al. 2010), partial nitritation-Anammox sludge (Yin et al. 2015) and Anammox sludge (Hou et al. 2015).

Besides the pure form of proteins and polysaccharides, those molecules also present in composite forms through covalent bonds. Higgins and Novak (1997) identified the lectin-like protein (i.e. binding capacity with sugar residuals from polysaccharide) in EPS extracted from activated sludge by means of sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) and amino acid sequencing approach. Gorner et al. (2003) observed a strong association of polysaccharide and protein through FT-IR analysis after fractionation by size exclusion chromatography (SEC). Such association was considered to be crucial in floc formation. Similar findings were observed by Garnier et al. (2005) in EPS extracted from different activated sludge plants. Park and Novak (2009) observed glycoprotein-specific lectin from activated sludge EPS and found that major lectin activities were present in hydrophobic region. Bourven et al. (2015) studied the protein fraction of EPS extracted from anaerobic granules by SDS-PAGE and far-west blotting. Glycoproteins were identified from periodic acid-Schiff (PAS) staining and were believed to originate from cell membrane and cell lysis. High molecular weight proteoglycan-like and sulfated proteoglycan-like compounds were found with the presence of sulfate and carboxylic groups. The composition and structure of these molecules had significant role in the mechanism of bioaggregates formation and stability which will be discussed in more detail in Chapter “Cell to cell interaction”. The composition and distribution of EPS inside the aggregates from different origin were found to be variable and internally heterogeneous, which depends on factors such as sludge type, operation status, wastewater composition, etc. (Liu et al. 2004b; Raszka et al. 2006; Sheng et al. 2010). Also different extraction methods could give different output of the EPS component analysis. The use of non-standard extraction method renders comparison infeasible (Bourven

et al. 2013; D'Abzac et al. 2010; Pellicer-Nàcher et al. 2013). Furthermore, all the reported EPS extraction methods were developed based on solubilization of the EPS matrix. However, a significant part of EPS which was believed to play an important role in cell aggregation is insoluble, such as the gel like polysaccharides (Seviour et al. 2012) and amyloids (Larsen et al. 2008). More detailed information on these compounds is discussed in Chapter "Cell to cell interaction".

Table 2.1 EPS composition in different systems

Aggregate type	Plant/reactor configuration	Substrate type	Protein	Polysaccharide	PS/PN	Others	Extraction method	Reference
Aerobic granules	Full scale SBR	Synthetic wastewater	253.8 mg/gVSS	20.8 mg/gVSS	0.079	-	EDTA	Li et al. (2014a)
Aerobic granules	Lab-scale SBR	Dye	0.109 mg/mgSS	0.040 mg/mgSS	0.22	Humic acid 0.051 mg/mgSS	CER[1)]	Gao et al. (2011)
Aerobic granules	Lab-scale SBR	Syntheticwastewater	163 mg/gSS	20-30 mg/gSS	0.12-0.18	-	-	Zhang et al. (2010)
Anaerobic granules	Full scale UASB	Brewery effluent	0.14 ±0.02 mg/mgDW	0.034 ±0.003 mg/mgDW	0.24	DNA 0.015 ±0.002 mg/mgDW	CER	Batstone and Keller (2001)
Anaerobic granules	Full scale UASB	Paper mill	87 mg/gDW	68 mg/gDW	0.78	Humic like substance 190 mg/gDW; uronic acid 7 mg/gDW; nucleic acid 10 mg/gDW	Sonication/ centrifugation	Metivier et al. (2013)
Anaerobic granules	EGSB	Distillery	101 mg/gDW	35 mg/gDW	0.35	Humic like substance 60 mg/gDW; uronic acid 5 mg/gDW; nucleic acid 21 mg/gDW	Sonication/ centrifugation	Metivier et al. (2013)
Anaerobic granules	Lab scale UASB	SO_4^{2-}/ethanol synthetic wastewater	56.6 ±1.5 mg/gDW	15.8 ±0.4	0.30	Humic like substance 36.5 ±0.5 mg/gDW; uronic acid 3.1 ±01 mg/gDW; nucleic acid 4.6 ±0.5 mg/gDW	CER	Guibaud et al. (2012)
Flocs	Conventional Activated sludge	Municipal wastewater	40.9% of total EPS	23.7% of total EPS	0.58	DNA 7.5 mg/mgVSS	Centrifugation/ heating	Ge et al. (2006)
Flocs	Conventional activated sludge	-	343 ±10	140 ±5	0.41	-	Sonication/ centrifugation	Guibaud et al. (2006)
Flocs	Conventional activated sludge	-	95 ±3	70 ±1	0.74	-	Sonication/CER	Guibaud et al. (2006)
Flocs	Conventional activated sludge	Domestic 70%; leachate 30%	46.2 ±1.2 mg/gSS	6.8 ±0.3 mg/gSS	0.15	-	Sonication/ thermal	Peng et al. (2012)

Flocs	Conventional activated sludge	Fruit process wastewater	14.8±0.6 mg/gSS	5.4±0.4 mg/gSS	0.36	-	Sonication/ thermal	Peng et al. (2012)
Flocs	Conventional activated sludge	Domestic 50% industrial 50%	45±3.6 mg/gMLSS	7.8±0.6 mg/gMLSS	0.17	DNA 14±3 mg/gMLSS	CER	Wilén et al. (2003)
Flocs	Conventional activated sludge	Domestic	45±6.5 mg/gMLSS	9.2±1.8 mg/gMLSS	0.20	DNA 4±5 mg/gMLSS	CER	Wilén et al. (2003)
Flocs	Conventional activated sludge	Leachate	28 mg/gMLSS	40 mg/gMLSS	1.43	DNA 7 mg/gMLSS	CER	Wilén et al. (2003)
Flocs	Conventional activated sludge	Oil refinery effluent	30±4.0 mg/gMLSS	5.7±1.1 mg/gMLSS	0.19	DNA 3.2±0 mg/gMLSS	CER	Wilén et al. (2003)
Flocs	Lab-scale SBR	Glucose	34.6% of total EPS	64.6% of total EPS	0.54	DNA 3.27 mg/mgVSS	Centrifugation/ heating	Ge et al. (2006)
Floc	Lab-scale SBR	Synthetic wastewater	4.6 mg/gSS	38 mg/gSS	8.31	-	Glutaraldehyde/ thermal	Hoa et al. (2003)
Flocs	Lab-scale CSTR	Organic chemical	48±3 mg/gVSS	17±4 mg/gVSS	0.35	DNA: 10±5 mg/gVSS	Glutaraldehyde	Sponza (2002)
Flocs	Lab-scale CSTR	Leather	47±3 mg/gVSS	27±2 mg/gVSS	0.54	DNA: 12±3 mg/gVSS	Glutaraldehyde	Sponza (2002)
Flocs	Lab-scale CSTR	Dye	42±5 mg/gVSS	26±3 mg/gVSS	0.62	DNA: 13±4 mg/gVSS	Glutaraldehyde	Sponza (2002)
Flocs	Lab-scale CSTR	Winery	70±3 mg/gVSS	17±2 mg/gVSS	0.24	DNA: 5.8±2 mg/gVSS	Glutaraldehyde	Sponza (2002)
Flocs	Lab-scaleAnammoxreactor	Syntheticwastewater	79.52 mg/gVSS	30.12 mg/gVSS	0.37	-	Heat	Yin et al. (2015)
Flocs	Anaerobic/oxic	Printing and dye wastewater	17.7±0.7 mg/gSS	3.9±0.2 mg/gSS	0.22	-	Sonication/thermal	Peng et al. (2012)
Flocs	Anaerobic/oxic/oxic	Paper and pulp wastewater	14.1±0.3 mg/gSS	2.9±0.2 mg/gSS	0.20	-	Sonication/thermal	Peng et al. (2012)
Aggregate [2)]	Anammox	Synthetic wastewater	67.9 mg/gVSS	25.3 mg/gVSS	0.37	-	CER	Hou et al. (2015)
Biofilm	Integrated fixed film activated sludge (IFFAS-4)	Raw sewage and primary effluent	70±2 mg/gDW	21±2 mg/gDW	0.3	Humic acid 53±5 mg/gDW; DNA 1.5±0.2 mg/gDW	CER	Mahendran et al. (2012)

Remark: 1) Cation exchange resin method (Frolund et al. 1996); 2) Not specified floc or granule

As mentioned above, EPS composition is dynamic from the same sludge at different stages or operational status. Thus the study of EPS composition pattern at different time could provide valuable information on the evolution of bioaggregate formation and the shift of process performance. For example, anaerobic condition or storage of activated sludge and aerobic granules could lead to a significant change in its EPS pattern. Liu et al. (2011) observed a drastic decrease in EPS amount of aerobic granules after 10 days storage. Nielsen et al. (1996) investigated the change of EPS component by quantitative analysis and SEC during the anaerobic storage of activated sludge. A significant decrease of total protein and total carbohydrate in EPS was observed through 12 days of storage. The UV detection, after EPS separation according to their size, revealed that the change of EPS compounds corresponded to the degradation of existing compounds. This might indicate that the decrease of protein and polysaccharide led to the release of gel material hence the deflocculation which finally caused the loss of dewaterability. Sponza (2002) observed a decrease of protein and polysaccharide during anaerobic condition of activated sludge leading to a higher sludge volume index (SVI). Settling condition is another operational factor which might change the EPS composition. Zhang et al. (2010) observed a decrease of SVI from 110 mg/L to 24-42 mg/L when settling time decreased from 39 min to 10 min for a nitrifying granular SBR. This was associated with a decrease of protein and no change of polysaccharide in granule EPS. Garnier et al. (2005) studied the molecular weight (MW) distribution of EPS using SEC and found that small MW EPS prevailed during poor settling condition. Miksch and Konczak (2012) followed a temporal study of EPS evolution during the life cycle of aerobic granules and found that protein concentration underwent a drastic increase in the formation period while polysaccharide concentration remained unchange. Seviour et al. (2009a) suggested that polysaccharides or glycosides were the gelling agent in aerobic granulation by studying the rheology of an EPS derived from aerobic sludge granules. It still remains arguable which component of EPS plays the major part in the bioaggregation process in flocs or granules. According to Seviour et al. (2009a), both protein and polysaccharide could form gels whereas polysaccharides possess a lower critical gelling concentration. And since protein and polysaccharide are cross-linked as composite, an attempt to differentiate these two could possibly be misleading (Seviour et al. 2009a). Thus a different view on focusing the mechanism of such cross links instead of finding which component plays the major role could be of interest in the study of bioaggregates. It could be found from the established correlation that the amount and composition of EPS response differently to operation conditions among

different processes. A more convincing conclusion could not yet be drawn until more detailed information, such as the type of cell to cell binding force, are revealed.

EPS distribution

The classic two-layer model of EPS structure (Fig. 2.4) was proposed by Nielsen and Jahn (1999). In this model, the inner layer which is denser and more concentrated and closely attached to the cell is defined as TB-EPS. The outer layer which is more dispersible and less concentrated, fading into the bulk is defined as loosely bound EPS (LB-EPS). The soluble part which is absolutely dissolved into the bulk solution is so called soluble EPS. However, this model describes the EPS structure outside the cells only in terms of "global" EPS while not mentioning their different biochemical components. It also fails to distinguish between flocs and biofilms.

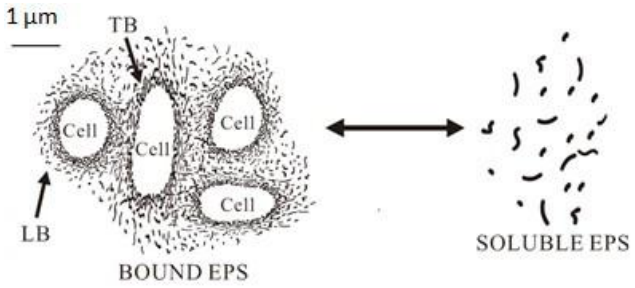

Fig. 2.4 Model of two-layer EPS structure proposed by Nielsen and Jahn (1999)

The use of microscopic techniques (e.g. scanning electron microscopy (SEM), CLSM), fluorescence staining and enzymatic degradation targeting different components of EPS and *in situ* staining technique allow the direct visualization of EPS distribution in the bioaggregates. Adav et al. (2008a) identified that protein and dead cells were located at the core while live cells and α-polysaccharide scattered at the outer rim of the aerobic phenol-fed granules through CLSM image. β-polysaccharides were distributed throughout the whole cell volume. Miksch and Konczak (2012) also found that proteins and dead cells were presented in the core of aerobic granule. However, the majority of β-polysaccharide was found in the core while the outer shell of the granules exhibited no fluorescence, presumably composed of α-polysaccharide. Calcium ion was found consistently distributed with protein and polysaccharide, which might imply the involvement of divalent cation bridging mechanism (Caudan et al. 2014; Higgins and Novak 1997; Sobeck and Higgins 2002). Similar observations were made in other studies (Chen et al. 2007; McSwain et al. 2005; Wang et al. 2005; Zhang et al. 2007). Proteins were always found in the core of the granules where cells were also presented in highest density. The large amount of protein might result from the

excretion of EPS which facilitated granulation; or might result from the release of cell material due to decay. The small discrepancies among different studies indicated the different types of cross-links which bound the macromolecules and cells. Those visual results could support the importance of EPS in the role of granulation and its stability. In the case of flocs, all EPS components were concentrated in the core while some polysaccharides were presented around filamentous fungi (McSwain et al. 2005). This is in accordance with the findings by de Beer et al. (1996) which showed that most EPS compounds were found in the core while no "coating" was observed. In the study of Szilveszter et al. (2013), polysaccharide was found distributed in all section of activated sludge flocs, indicating its importance in sludge flocculation.

2.3 Cell to cell interactions

Cell aggregation is a dynamic process which results from the combination of all physico-chemical and biological activities. To understand the mechanism of cell aggregation, it is a prerequisite to investigate the forces driving cell to cell interaction. The studies on cell physico-chemical interaction were basically derived from colloid and surface chemistry whereas biological interactions could range from genetic to physiological levels and are species dependent. The driving forces discussed in this section are applicable to cell to cell interactions of all types of bioaggregates while the cell to carrier interaction for attached growth system is not covered.

2.3.1 Physico-chemical interactions

Rooted from colloidal and surface chemistry, the classic *DLVO* and *extended DLVO* theory have been adopted to describe the cell to cell interactions as well as flocculation process. According to classic DLVO theory, when particles approach each other, the double layer is compressed. When a certain distance is reached, the van der Waals force exceeds the repulsive electrostatic force and the second minimum energy is reached. That means a reversible aggregation with a fractal structure as described in Section 2.2 is achieved (Yuan et al. 2014). The extended DLVO theory, in which the hydrophobicity interaction is additional to the classic one, was then considered to be the basic theory of cell aggregation (Hermansson 1999; Olofsson et al. 1998). In the extended DLVO theory, the following four interactions are

considered: (i) *van der Waals forces* (generally attractive), (ii) *repulsive forces from the electric double layer* surrounding the cell, (ii) *hydrophobic/hydrophilic interactions* and (iv) *osmotic interactions* (negligible for cell particles). According to the theory, when the overall Gibbs energy contributed by all components is negative, cells tend to adhere to each other or to a surface. The repulsive force from the electric double layer is strongly affected by ionic strength. The addition of cations in the bulk solution resulted in an increase of ion strength which compressed the thickness of the double layer leading to the decrease of the repulsive force (Sobeck and Higgins 2002). Li et al. (2012) found that the flocculability of activated sludge was enhanced by addition of Al^{3+} and Fe^{3+} which could be explained by the extended DLVO theory. It was also hypothesized that the soluble EPS and bound EPS extracted from activated sludge resembled the Stern's layer and diffusion layer in the double electric layer model (Yu et al. 2009; Yuan et al. 2014). According to Liu et al. (2004a), cell hydrophobicity was believed to be the fundamental force in bioaggregation explained through a thermodynamic interpretation. Furthermore, the hydrophobic EPS has significant contribution. Cell hydrophobicity was also believed to be a triggering force for granulation in its initial phase (Liu et al. 2004a). Zhang et al. (2007) studied the relative hydrophobicity during sludge granulation from aerobic flocs and found that granules had higher cell hydrophobicity than flocs and had a high degree of correlation of 0.969 with the protein to polysaccharide (PN/PS) ratio. Hou et al. (2015) studied the surface properties of Anammox granules through quantitative analysis based on extended DLVO theory and found that hydrophobic interaction was the main driving force in the aggregation. Furthermore, the amino acid composition and protein secondary structure from extracted EPS were analyzed. It was discovered that the high hydrophobicity of Anammox sludge was contributed by the presence of highly hydrophobic amino acids and by a loose protein secondary structure facilitating a full exposure of the inner hydrophobic groups (Hou et al. 2015).

Other important inter-particle forces include hydrogen bonds, thermodynamic forces, covalent bonds etc. Mayer et al. (1999) attempted to rank the contribution of binding forces within biofilms and found that electrostatic and hydrogen bonds were the dominating forces. The extended DLVO theory could quantitatively predict the non-bio particles; however, it has limited applicability in bioaggregates when microbial activities, especially the excretion of EPS, affect the cell surface characteristics (Hermansson 1999). Furthermore, gelation, divalent cation bridging and lectin binding have been widely investigated in search of mechanisms of cell aggregation, all of which are physico-chemical interactions but involve and base on the production and function of EPS. Thus those EPS related forces are considered

to be microbial interactions in this study. It should be emphasized that microbial activities such as surface dehydration caused by proton pump and secretion of EPS could significantly affect or even trigger the cell to cell interactions (Hermansson 1999). In addition the hydrophobicity of the cell surface may partly be due to the hydrophobic fraction of EPS macromolecules.

2.3.2 EPS mediated microbial interactions

Biological interaction of cells could be understood as interactions mediated by microbial activities. The most important microbial activity which leads to cell aggregation is the production of EPS, based on which various theories have been developed attempting to explain the mechanisms.

In the *divalent cation bridging theory*, divalent cations bind with negatively charged functional groups of EPS macromolecules and strengthen the structure of the EPS matrix of the activated sludge flocs (Higgins and Novak 1997). The process was well modelled by Higgins and Novak (1997), in which negatively charged sites especially on proteins were bridged by divalent cations, thus a stable EPS network was formed which connects the cells into aggregates. The model was validated by two case studies of direct addition of Ca^{2+} and Mg^{2+} to a pilot scale and full scale WWTP in which positive effects from the addition on nutrient removal and dewaterability were observed (Higgins et al. 2004a; Higgins et al. 2004b). Sobeck and Higgins (2002) found that the divalent cation bridging theory could best explain the flocculation process among the other two theories, namely alginate and DLVO theory, in explaining the role of cations in sludge flocculation. Similar to flocs, divalent cation bridging was considered one of the main mechanisms in granule formation (Liu et al. 2010a; Zhang et al. 2007). Caudan et al. (2014) conducted a test on enzymatic hydrolysis on aerobic granules targeting different EPS components followed by shear tests. The results showed that macro-particles detachment under shear occurred after α-amylase hydrolysis and soluble/micro-particles detached after protein hydrolyzed by savinase. The simultaneous release of calcium after savinase treatment gave evidence that cation bridging anionic protein was prevailing in maintaining cell to cell level structure. This is consistent with the study of Higgins and Novak (1997) who found that divalent cations bind more extracellular protein than polysaccharide. Recently Bourven et al. (2015) identified the presence of sulfate groups ($-SO_3^-$) and carboxylic groups ($-COO^-$) in proteoglycan-like substances purified from bound-

EPS of anaerobic granules. This might further facilitate bridging with divalent cations such as Ca^{2+} which was found to be presented in high concentration.

In *alginate theory*, bacteria produce alginate-like exopolysaccharide which is composed of mannuronic and glucuronic acid arranging as a linear polysaccharide chain without branches. In the presence of Ca^{2+}, the poly(glucuronic acid) blocks are buckled and cross linked with Ca^{2+} and form the "egg-box" structure which has gelling property thus binding cells together (Sobeck and Higgins 2002). This alginate-like polysaccharide was found in both flocs and granules (Lin et al. 2013; Yuan et al. 2014). Alginate-like exopolysaccharides from aerobic granules was extracted and analyzed by Lin et al. (2010) and was considered as one of the dominant exopolysaccharide and contributes to the hydrophobicity, gelling property and cross linking of divalent cations. Li et al. (2014b) proposed that gelation was the main mechanism of aerobic granulation after successfully cultivated artificial granules at high concentration of alginate. However, alginate is not the only polysaccharide with gel-forming property. Seviour et al. (2009b) applied a novel rheological approach which identified aerobic granules as *hydrogels*. By definition, hydrogels, which are composed of cross-linked polymer matrices, are gels that swell and de-swell. Later Seviour et al. (2009a) found that the polysaccharide content of EPS from aerobic granules exhibits sol-gel transition at pH 9. The specific polysaccharide was purified (Seviour et al. 2010a) and its structure was elucidated by Nuclear magnetic resonance (NMR) spectroscopy (Seviour et al. 2010b). The molecule, which is named as "*granulan*", was described as a complex branched heteropolysaccharide (Seviour et al. 2012). This approach provides insight in understanding the mechanism of EPS cross-links at molecular level. More candidates exhibiting gelling property are expected to be discovered.

Higgins and Novak (1997) identified the lectin-like protein in the EPS from activated sludge and confirmed its role in binding polysaccharides to form cross-linked exopolymers. Later Park and Novak (2009) conducted lectin assay on EPS extracted from activated sludge and proposed that *lectin mediated aggregation* is one of the mechanisms for sludge flocculation. Such protein/polysaccharide composite forms in EPS was discovered both in activated sludge (Garnier et al. 2005; Gorner et al. 2003; Park and Novak 2009) as well as in anaerobic granular sludge (Bourven et al. 2015).

Except the hydrogel approach, all the above theories are based on solubilization based EPS extraction and characterization methods. However, some components which are believed to play key role in cell aggregation are insoluble thus overlooked. Besides alginate and granulan, *amyloid adhesions* were found to play diverse functions in activated sludge flocs

(Larsen et al. 2008). In medical science, amyloid fibrils are misfolded proteins or polypeptides causing Parkinson's and Alzheimer's disease (Serpell 2000). Amyloids produced by bacteria share similarities as thin fibrils, insoluble, and high resistant to denaturants. Various microcolony-forming denitrifiers and PAOs, including *Thauera*, *Azoarcus*, *Zoogloea* and *Aquaspirillum*-related microorganism were found to produce amyloids. Some filamentous bacteria belonging to *beta-*, *gamma-proteobacteria*, *bacteroidetes* and *chloroflexi* also express amyloid adhesins. Amyloids were found in relatively large fraction of EPS in microcolonies and filaments and were believed to increase surface hydrophobicity of flocs (Larsen et al. 2008). However, the studies on amyloids were mostly conducted on pure culture such as *E. coli* and *Salmonella enterica* while that of mixed culture sludge is quite limited (Chapman et al. 2002; Szalai et al. 2000). It is expected that more researches will be conducted on these insoluble part of EPS.

2.3.3 Other microbial interactions

Proton translocation-dehydration theory was firstly proposed by Tay et al. (2000) and validated through anaerobic granular sludge from a UASB reactor by Teo et al. (2000). In brief, the adhesion of cells is initiated by the dehydration of bacterial surface, which is the result of surface protonation through proton pumps on the cell membrane. According to Liu et al. (2003), the proton gradient of aerobic bacteria was achieved by electron transport system (ETS) to transport process electron to external acceptor, i.e. oxygen, for adenosine tri-phosphate (ATP) synthesis. In anaerobic bacteria such as methanogens, ATP was synthesized by electron transport and oxidative phosphorylation in the absence of oxygen. Thus the theory seems applicable for both aerobic and anaerobic bacteria regardless of type of substrate and operation conditions. However, with limited literature and lack of experimental support, the surface hydrophobicity caused by proton translocation dehydration was "outcompeted" by other factors.

Quorum sensing (QS) is an important concentration based cell to cell communication mechanism. By definition, QS bacteria produce and release signal molecules to sense cell density and activate gene expression (Shrout and Nerenberg 2012). One of the important signal chemical N-acylhomoserine lactones (AHLs), produced by gram-negative bacteria, was proved to play significant role in biofilm formation (Feng et al. 2013; Ren et al. 2010). Lv et al. (2014a) found AHL in both activated sludge and aerobic granular sludge, in which the high

concentration in granular sludge was correlated with a higher EPS amount. Several bacterial genus was studied and proved to be able to produce AHL which triggered the gene expression for biofilm formation and EPS production (Shrout and Nerenberg 2012). For example, *Aeromonas*, *Pseudomonas* and *Vibrio* could produce AHL or autoinducer (AI) to initiate biofilm formation (Lynch et al. 2002). *Pseudomonas* and *Xanthomonas* produce AHL and diffusible signal factor (DSF) respectively to trigger EPS production (He and Zhang 2008; Williams and Cámara 2009).

2.4 Mechanisms of bioaggregates formation

Step wise flocculation mechanism is rare in literature which may lead to the assumption that flocculation is random process mainly governed by physico-chemical adhesion. However, more complicated biological activities were revealed to compose the microbial background of flocculation. The section herein attempts to postulate a stepwise flocculation process which encompasses different physico-chemical and biological forces driving flocculation. Also different theories of granulation mechanisms are summarized and compared aiming at providing a comprehensive understanding on the granule formation which leads to different granule structure and its potential implication in application.

2.4.1 Flocculation

Raszka et al. (2006) proposed three different theories, namely DLVO theory, alginate theory and divalent cation bridging theory to explain the flocculation process. However, the trigger of flocculation and the actual mechanism still remain unclear. Bossier and Verstraete (1996) argued that environmental signals such as substrate gradients, feast famine feeding strategy, chemical or physical stresses and predation could encourage microorganisms to aggregate. Those signals trigger the adaptation of cell metabolism which favors aggregation, for example, microorganisms tend to be more hydrophobic during starvation (Kjelleberg et al. 1987). However, a systematic flocculation process theory has not yet been developed. Based on the currently known theories, the following flocculation mechanism is proposed:

(i) Homogeneously distributed free cells sense the environmental stress as described by Bossier and Verstraete (1996).

(ii) Gathering of free cells either spontaneously or through quorum sensing by quorum

producing bacteria.

(iii) When signal molecule reaches the threshold concentration, genes encoding microcolony or floc formation related activities are activated, such as the production of EPS and the change of surface characteristics.

(iv) Free cells and microcolonies adhere to each other through physico-chemical interaction and amplify into the fractal structure.

(v) Mature flocs are formed when a steady state of attachment-detachment of dispersible fraction is reached.

The proposed mechanism is based on the assumption that the cells are under continual environmental change or stress which discontinuously gives signals for cell aggregation. Activated sludge system is continually subject to environmental stress due to WWTP operation, such as aerobic/anoxic conditions, fluctuation of influent substrate concentrations, substrate concentration gradient in plug flow system, etc. Thus such dynamic system is favourable for the floc formation.

2.4.2 Granulation

In contrast to flocculation, granulation is a more complicated process in which a higher energy barrier shall be overcome to achieve a compact and dense structure. Based on the image of granules in different growth stages, several mechanisms were postulated by various authors. Although published in earlier years, Liu et al. (2003) proposed a most complete four-step granulation model (Fig.2.5) which attempted to cover as much as possible the known mechanisms. Briefly the four steps are:

Step 1: physical movement to initiate cell to cell or cell to nuclei contact which involves hydrodynamic force, diffusion force, gravity force, thermodynamic force and cell mobility.

Step 2: Initial attractive force to stabilize cell to cell contacts. This involves various physical, chemical and biochemical forces, including the DLVO theory, hydrophobicity, hydrogen bond as well as filamentous bridging.

Step3: Microbial forces to make mature aggregation. This includes the EPS excretion, cellular cluster growth, metabolic change and genetic competence induced by environment.

Step 4: Steady state granulation induced by hydrodynamic shear forces.

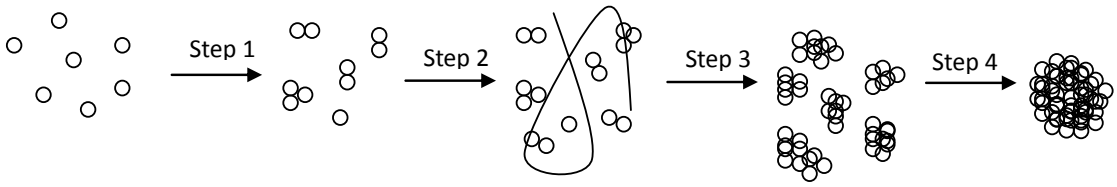

Fig. 2.5 Demonstration of the four-step granulation model proposed by Liu et al. (2003)

This model was based on the hypothesis that shear force played an essential role in the formation of granules because free swimming cells were more accessible to substrate thus the exclusion of those small particles ensured the nutrient availability for granular bacteria (Liu and Tay 2002). This model was adopted to explain the granulation mechanism of aerobic granules cultivated from activated sludge (Lv et al. 2014b). According to the author, granulation started from *step 3* when activated sludge was used as seeding sludge in which microbial forces were achieved by feast-famine condition in SBR. Hydrodynamic shear force was achieved by high bubbling of oxygen in *step 4*. The four steps model was especially in good compliance with filamentous or ciliate backbone granulation theory in which a high shear stress allowed the detachment and washout of filaments. For example, Wang et al. (2006) proposed a five-stage granulation mechanism which includes: (i) microbe multiplication phase when bacteria formed *zoogloea* or mycelia pellets, (ii) floc appearance phase when small flocs of 0.3-0.8 mm diameter were formed, (iii) floc cohesion phase when small flocs were entrapped by filamentous microbes or EPS to form mature flocs, (iv) mature floc phase whenflocs with diameter of 3.0-6.5 mm were formed and (v) aerobic granule phase whenfilaments were detached due to shear stress and compact granules were formed. Beun et al. (1999) studied the granulation of sludge from an organic carbon removal SBR and suggested that fungi initially became dominant and filamentous mycelia pellets comprised the biomass during the start-up. Filaments were then detached due to shear stress and the pellets became compact and smooth. The pellet acted as a matrix on which bacteria immobilized and grew out as colonies. Due to oxygen or substrate limitation, the pellet disintegrated and bacterial microcolonies further grew into compact granules and became dominant in the reactor. Gonzalez-Gil and Holliger (2014) observed the process of microbial clusters of *Accumulibacter* mono-colonies rooting from the interior of the granules with filaments initially served as backbone, which indicated biomass outgrowth as the main force for granulation and that the presence of few filaments only existed at less shear region.

However, recent studies showed that hydrodynamic selection might not be the necessary factors leading to granulation. For example, Weissbrodt et al. (2013) found that clustered

Accumulibacter could be formed spontaneously under steady state even though three time slower than under wash-out conditions. Barr et al. (2010) also found that granule formation and segregation was not the result of selection pressure for (phosphate accumulating organisms) PAO organism *Candidatus Accumulibacter phosphatis*. In their study, both microcolonies outgrowth into dense homogeneous granules and microcolonies aggregation into clustered irregular structure were observed. Vlaeminck et al. (2010) studied the growth of oxygen limited autotrophic nitrification/denitrification (OLAND) granules which anaerobic ammonium oxidizing bacteria (AnAOB) composed the core and aerobic ammonium oxidizing bacteria (AerAOB) formed the rim grown in SBR in which the layered OLAND granules were obtained at low settling rate. It was suggested that small granules were formed through aggregation and expected to expand due to microbial growth as similar to other aerobic and anaerobic granules. Besides the disintegration fate, large granules could also replicate through a budding pathway and division pathway. The cause of this replication was believed to be the limitation of substrate transfer with the increase of granule size. Also Wan et al. (2011) concluded that high superficial air velocity and low settling time were not necessary to produce aerobic granules after studying the evolution of granulation in a sequencing batch airlift reactor.

Random coagulation was also proposed by different authors yet in minority. Zhou et al. (2014) proposed a dynamic granulation process which was composed of four steps, namely initial aggregation, granule growth caused by floc attachment, another self-aggregation domination and re-granulation. The author claimed that the floccular sludge at steady stage was the result of the dynamic attach-detachment process rather than incapability of granulation. Thus the granulation was a random coagulation process. Similarly, Liu et al. (2010d) observed "no strong microbial selection" after granules formed from activated sludge. However, this approach was negated by Lv et al. (2014b) who studied the microbial communities of seeding flocs and sliced aerobic granules. The granulation resulted in layered microbial structure with mainly *Thauera* in the core and *Microbacteriaceae* distributed on the surface layer. The shift of community from flocs to granules and distribution within the granule depth suggested that "granules are formed via a deterministic rather than via a random aggregation-disintegration mechanism" (Lv et al. 2014b).

2.4.3 Consideration on the bioaggregation mechanisms

Stepwise floc formation mechanisms were rarely found in literature probably because sludge flocculation is similar to the coagulation of other non bioparticles such as protein coagulation or the primary flocculation process prior to activated sludge process in WWTP. Thus colloidal and surface chemistry was predominantly selected to explain its mechanism. However, microbial activities such as bacterial growth/decay, EPS production, substrate loading and growth of filaments render the flocculation more complicated than the non-bio flocculation. Because those microbial activities change the surface characteristics and the flocculation/deflocculation process becomes dynamic. In the view of application in wastewater treatment, it is important to understand the general flocculation process as well as its mechanism at microbial level so that a good flocculation and treatment performance could be achieved through operational strategy.

The above discussed granulation models provided a comprehensive overview which is valuable in both fundamental and engineering aspects. It is certain that granulation is not the result of a single aggregation process by physico-chemical forces but also biological evolutional processes involving growth, replication and selection at both cell and granule level. However, the existing models could not explain the reason for different granular structures. For example, both clustered (Gonzalez-Gil et al. 2001) and layered structures (Zheng et al. 2006) could be formed by anaerobic granules performing anaerobic digestion process. Similarly, PAO organisms could form homogeneous and clustered granular structure simultaneously within one reactor (Barr et al. 2010). According to Gonzalez-Gil et al. (2001), clustered structure was associated with space limitation and low substrate concentration while compact layered structure was associated with growth rate limitation and high bulk substrate concentration. It is reasonable to hypothesize that the choice of granular structure is decided by the group of microorganisms to optimize the mass transfer, juxta-position and syntrophic association. As mentioned above, microorganisms can “sense” the environmental signal which triggers genetic expression such as interspecies quorum sensing. Interspecies quorum sensing was observed and proved by Zhang et al. (2011) who suggested that “QS as a novel regulation target for the biogranulation process”. Thus interspecies quorum sensing could be a candidate for the mechanism of granular structural selection. In granule based wastewater treatment process, a fast cultivation of dense granules with good settling is important from a time and cost efficient point of view. Thus the hydrodynamic selection based mechanism is preferred whenever applicable because it allows the fast selection of large and dense granule. However, the fast granulation is achieved at the cost of wash out of active biomass of smaller

size. Since hydrodynamic shear was proved not to be always necessary for granulation, a choice between biomass retention and fast granulation should be carefully considered.

2.5 Role of EPS in bioaggregates stability

The fundamental differences in structures and mechanisms imply that the EPS produced from flocs and bioflm shall show significant differences. However, both bioaggregates share similarity in global composition, i.e. proteins, polysaccharides, humic-like substances and nucleic acids. More detailed studies on the characterization of exopolysaccharide and extracelluar protein showed their molecular variations which led to the difference binding strength of the two bioaggregates (Lin et al. 2013; Lv et al. 2014a; Mart ń-Cereceda et al. 2001; Seviour et al. 2009a; Zhang et al. 2015a). However, the approaches for studying the effect of EPS in flocs and biofilms were quite different. For flocs, the layered EPS model was mostly applied in establishing the correlation between floc stability and LB-EPS and TB-EPS. Whereas in the study on granule stability, test on enzymatic degradation on certain compounds of EPS and direct visualization under CLSM after selective staining were most frequently applied. In this chapter, separate discussions on the role of EPS on flocs and biofilms will be given followed by comparison of floc EPS and biofilm EPS. It should be mentioned that EPS is not the sole factor even may not be the main factors which maintains or degrades the bioaggregate stability. As mentioned above, granulation is a deterministic process which is triggered by environmental change and preceded by microbial activity. Thus the loss of bioaggregate stability could also be a survival strategy for bacteria under environmental stress. For example, disintegration may occur under starvation condition to facilitate the interior microorganisms to access substrate. Filamentous bulking is one of the major causes for deflocculation, which is not related to EPS.

2.5.1 Floc

The influence of EPS from flocs have been acknowledged and extensively studied with the implication of flocculation, settleability and dewaterability (Liu and Fang 2003). However, early studies did not take into consideration of the stratification of the EPS, which was further categorized into TB-EPS and LB-EPS. Literatures showed that the analysis of “stratified”

EPS could give correlative information with floc stability related process performances (Table 2.2).

Table 2.2 Studies on stratified EPS by different extraction methods

Sludge source	Extraction method	Composition and correlation	Reference
Activated sludge	**Supernatant**: Settling for 1.5 hour at 4 ˚C; **Slime**: centrifugation at 2000 g for 15 min at 4 ˚C; **LB-EPS**: further centrifugation at 5000 g for 15 min at 4 ˚C; **TB-EPS**: ultrasonication at 20 kHz and 480 kW for 10 min then centrifugation at 20,000 g for 15 min at 4 ˚C	Increased PN and PN/PS ratio in supernatant, slime and LB-EPS is correlated to decreased dewaterability; PS in any EPS layer shows no correlation with dewaterability; PN, PN/PS in TB-EPS shows no correlation with dewaterability.	Yu et al. (2008)
Activated sludge	**LB-EPS**: vortex in falcon tube in 50 ˚C warm water for 1 min and centrifugation at 4000 g for 10 min; **TB-EPS**: incubation at 60 ˚C for 30 min and centrifugation at 4000 g for 15 min.	LB-EPS plays unfavorable role in flocculation settling, compression and dewatering. No correlation between TB-EPS and process performance parameters.	Yang and Li (2009)
Activated sludge	**Slime**: centrifugation at 2000 g for 15 min at 4 ˚C; **LB-EPS**: further centrifugation at 5000 g for 15 min at 4 ˚C; **TB-EPS**: ultrasonication at 20 kHz and 480 kW for 10 min then centrifugation at 20,000 gfor 15 min at 4 ˚C	Sludge after LB-EPS extraction has the highest d_f value which implies the key role of TB-EPS in the floc structure; TB-EPS and sludge after LB-EPS extraction shows strong gel like and compact structure, which may indicate the key role of TB-EPS in the gel like characteristic and drying process of sludge.	Yuan et al. (2014)
Activated sludge	Sludge exposed to shear intensity of 800 l/s by mechanical stirring with a flat paddle mixer for 0-250 min. **Readily extractable EPS** are released to solution after infinite time of shear exposure. The remaining is the **stable part**. EPS are extracted by CER methods	PN/PS of readily-extractable EPS fraction is 0.22 which indicates carbohydrates as predominant part; Dispersible fraction of EPS accounts for 18%;	Sheng et al. (2006)
Anaerobic methanogenicfloc		PN/PS of readily-extractable EPS fraction is 2.66:1 which indicates polysaccharide as main part; Dispersible fraction of EPS accounts for 34%.	
Activated sludge and anaerobic methanogenic sludge	**LB-EPS:** ultrasonication for 2 min followed by horizontal oscillation for 10 min, repeat ultrasonication for 2 min **TB-EPS**: re-suspended pellet mixed with CER and stirred at 4 ˚C for 12 hours	Both LB-EPS and TB-EPS have substantial contribution, while LB-EPS displays positive role in sludge aggregation	Liu et al. (2010c)
Biofilm and suspended sludge from a sequencing batch biofilm reactor	**S-EPS [1]:** ultrasonication at 20 kHz and 40W for 30s, followed by centrifugation at 2000 g for 15 min at 4 ˚C **LB-EPS:** horizontal vibration for 1 hour followed by centrifugation at 5 g for 15 min at 4 ˚C	The contribution of combined S-EPS and LB-EPS was 23% for suspended sludge while negligible for biofilm; The contribution of LB-EPS and TB-EPS was 16% and 30% for suspended sludge. The contribution of LB-EPS and TB-	Zhang et al. (2014)

	TB-EPS: resuspended pellet mixed with CER and stirred at 600 rpm for 1 hour at 4 ˚C	EPS was negligible for biofilm.

Remark:

1) EPS extracted from suspended sludge

Based on a layered structure, EPS on different layer was believed to play their specific roles in maintaining different parts of the floc structure. Because flocs are shear sensitive, the separation of EPS on different parts of the floc is normally categorized by some empirical extraction methods which presumably correspond to different connection strength. Table 2 gives some examples on the study of layered EPS, in which most of the authors found a negative correlation between LB-EPS and sludge flocculability and dewaterability with the exception of Liu et al. (2010c) and Zhang et al. (2014). Such discrepancy was considered due to the different extraction method as well as an ambiguous definition between LB-EPS and TB-EPS (Wang et al. 2014a). The authors proposed a new classification of EPS between floc level and microcolony level. It was found that EPS at microcolony level had both higher total amount (60% at microcolony level and 40% at floc level) and PN/PS ratio (2.9 vs 2.2). Also EPS at microcolony level had higher molecular weight and more hydrophobic. They concluded that cation bridging interaction was more important at floc level because CER method could not release EPS from microlonies, while polymeric entanglement and hydrophobicity interactions were more important at microcolony level. Thus the classic DLVO theory seemed more applicable at floc level than microcolony level. The classification is consistent with the fractal model of flocs (Fig. 2.2), in which basic units (microcolonies) were “loosely bound” to each other and reversible while cell to cell bound forming microcolonies were more “tightly bound” and irreversible (Yuan et al. 2014). However, without consideringthe microbial species or group specific cell aggregation mechanism, the conclusion might be over generalized. Klausen et al. (2004) proposed that different microcolony forming bacteria had different type and strength of aggregation force. It was found that colony strength of *Firmicutes* were ensured by hydrophobic interaction, presumably from hydrophobic EPS, while that of *Gamma-proteobacteria* and *Bacteroidetes* colonies were bound by divalent cation bridging with EPS, and *Delta-proteobacteria* colonies were bound by both forces. Among the studied bacteria, *Beta-*, *Gamma*and *Delta-proteobacteria* had the highest colony strength, followed by *Firmicutes* and *Bacteroidetes*, and *Alpha-proteobacteria* was the weakest. At floc level, the cell to floc adhesion strength was also different among different groups (Wilen et al. 2008). It was observed that *Gamma-proteobacteria* formed strong microcolonies but was most weakly bound to flocs when it was

under shear stress. Nielsen et al. (2003) also found different floc forming bacteria respond differently to shear as well as under anaerobic conditions. Thus more attention should be focused on the microbial background of the biomass which gives different expression of floc and microcolony forming properties and EPS production.

2.5.2 Granule

Extensive engineering researches were focused on the maintenance of granular stability as well as its performance in wastewater treatment functions. The main engineering factors contributing to aerobic granular stability were summarized by Khan et al. (2013), which included substrate composition, organic loading rate, hydrodynamic shear force, DO concentration, settling rate, aerobic starvation, food to microorganism ratio, configuration of the reactor, hydraulic retention time, volumetric exchange ratio, pH, temperature, chemical toxicity, etc. According to Liu et al. (2004b), it was the change of environmental conditions that triggered bacteria in shifting microbial community and re-regulating metabolic pathway of EPS production. Furthermore, the lifecycle of the cells in granule may change the EPS as well as the granule structure over time. For example, the death of cells may result in the release of cell material and leave cavities in the granule. The microbial activity inside granule is dynamic in response to environmental change so as the EPS. The change of EPS, which could be total amount, composition and/or physico-chemical characteristics, subsequently influences the status of granular structure and stability. The sub-section herein focuses on the involvement of EPS in granular stability.

Quantitative determination and *in situ* visualization of EPS compound after selective staining were adopted by various authors to investigate the correlation between EPS composition/distribution and granular stability. McSwain et al. (2005) concluded that the stability of an activated sludge granule depended on a noncellular protein core based on the observation that (i) PN/PS ranging from 6-8 in all the samples tested and (ii) the CLSM image showed that core was mainly comprised of proteins while cells and α-polysaccharides located at the out rim of the granule. However, β-polysaccharide was not stained and no correlation studies between EPS components and surface characteristics (hydrophobicity and surface charge) were conducted. Thus the conclusion is not sufficiently convincing. Similar observation have been made by Chen et al. (2007) who conducted a quadruple staining on all of the EPS components including β-polysaccharide on an acetate-fed aerobic granule. Indeed,

the distribution of proteins, cells and α-polysaccharides was consistent with McSwain et al. (2005) but β-polysaccharide was also found to spread throughout the interior of the granule. Considering the rigid molecular structure of β-polysaccharide, it is reasonable to presume its role in the entire granular structure. Zhang et al. (2007) found that PN/PS increased from 2.3 to 4.9 with the granulation process for glucose and acetate fed aerobic granules. A higher PN/PS ratio corresponded to a higher surface hydrophobicity and a lower surface charge was believed to contribute to a more stable structure (correlation coefficient 0.969). Therefore it was concluded that protein may play major role in granular stability. However, since no direct imaging was conducted, the location of protein could not be confirmed and its relationship with other EPS components and cells could not be established. Miksch and Konczak (2012) proposed that the crucial role of protein in granular stability was based on its high correlation with hydrophobicity (0.97) and that the concentration of protein increased by 5 folds while that of polysaccharide remained unchange during granulation. The coincident location of proteins, β-polysaccharides and calcium ions concentrated in the coremight support the theory of divalent cation bridging of macromolecules. Adav et al. (2008a) found that enzymatic hydrolysis of β-polysaccharides decreased the stability of a phenol-fed aerobic granule while disintegration of the granule did not occur when proteins and α-polysaccharides were hydrolyzed. This leaded to the conclusion that β-polysaccharides formed the backbone of entangled EPS macromolecules which maintained the stability of the granule. The findings of Adav et al. (2008a) were consistent with an earlier study by Wang et al. (2005), in which only β-polysaccharide was stained and studied. The high hydrophobicity and low biodegradability of the β-polysaccharides observed in the outer rim of an acetate-fed aerobic granule led to the conclusion that this part of insoluble β-polysaccharides played a protective role in maintaining the granule stability. However, a different result was obtained by Caudan et al. (2014) in which treatment of α-amylase led to a more serious disruption than the treatment of β-amylase from aerobic granules fed by different organic substrate. Indeed, the arguments which were based on the use of β-amylase might underestimate the role of α-polysaccharide, which could also be hydrolyzed by β-amylase from its non reducing end (Doelle 1994). However, it is reasonable to assume that the rigid linear structure of β-polysaccharide may play some role in maintaining the structure of EPS matrix as well as the stability of the granules.

Correlation studies between different EPS components and granule stability as well as granulation process might provide some information in the role of EPS, however, it also has its limitations. For example, solubilization based studies are not always reliable since it is likely that multi-component biopolymers are insoluble. *In situ* approaches give no information

about molecular structures and intermolecular interactions (Seviour et al. 2009b). The composition/distribution of EPS as well as the granular structure is process dependent thus granules taken from their specific living environment may have different microbial physiology and a biased conclusion could possibly be drawn. Also comparison of different studies is questionable due to their different source of origin as well as extraction methods. Furthermore, the well-established correlations could not reveal the real mechanisms behind the cell granulation which distinguishes it from flocculation. To overcome the above limitations, a novel approach which identified aerobic granules as *hydrogels* was proposed by Seviour et al. (2009b), in which aerobic granules were proven to be hydrogels through rheological study. By applying the same enzymatic degradation targeting different EPS components as Adav et al. (2008a), it was found that the degradation of proteins and α-polysaccharides led to the loss of storage modulus *G'* which implied their major role in the maintenance of granule stability. This result was in contrast with Adav et al. (2008a) who claimed that β-polysaccharide plays the main role. The information revealed could be valuable in further formulating process strategies in wastewater treatment application targeting at a better biodegradation of pollutants and biomass retention performances.

2.5.3 Key EPS molecules identified to differ floc and biofilm

As mentioned above, EPS from flocs and biofilms share similarity in global composition while differ in detailed structure of each component. A few examples on the comparative study on EPS extracted from flocs and biofilms are given below. Granulan and alginate-like polysaccharide are two identified gel forming exopolysaccharide. Seviour et al. (2009a) observed a reversible sol-gel transition at pH 9.0-12.0 in EPS extracted from PAO granules while the same phenomena did not occur in EPS extracted from flocs. Lin et al. (2013) found that alginate-like exopolysaccharide existed in EPS extracted from both flocs and aerobic granules, in which the exopolysaccharide from granules contained more poly(guluronic acid) block than poly(guluronic acid-manuronic acid) block while for that extracted from flocs equal number of poly(guluronic acid) block and poly(guluronic acid-manuronic acid) block were found. It was believed that the structural difference in the specific alginate-like exopolysaccharide led to the significantly stronger gelling property of granules. Earlier, Mart ń-Cereceda et al. (2001) found that extracellular protein in biofilm from a rotating biological contactor was 3.5 times higher in concentration than that in suspended activated

sludge floc. Also a higher hydrophobicity of the biofilm EPS was believed to contribute to the compact biofilm structure. Recently, Zhang et al. (2015a) observed that a higher percentage of extracellular protein related to catalytic activity was found in flocs than in granules. Specifically the presence of hydrolase in the flocs may cause the degradation of EPS matrix and subsequently resulted in a loose floc structure. Lv et al. (2014a) found more proteins and polysaccharides in the hydrophobic fraction of EPS extracted from aerobic granule than activated sludge flocs. Besides, a significantly higher QS molecule AHL was found in granules than in flocs. Furthermore, some hydrophobic bacterial such as *Flavobacterium* existed only in granules. Thus the differences in the structure of EPS components, production of QS molecules as well as microbial communities contributed the structure of bioaggregates.

2.6 Summary and perspectives

This review has outlined and updated the knowledge on different aspects of bioaggregates, including their physico-chemical and microbial structure, aggregation mechanism and stability with special focus on the role of EPS. The differences among flocs, granules and attached growth systems have also been discussed. There are fundamental differences in structures among bioaggregates whereas generalized study on "aggregates" may not be proper with respect to their different structures and formation mechanisms. Flocs are fractal and unstable bioaggregates which are under frequent flocculation-deflocculation in wastewater treatment bioreactors while granules are independent "individuals" which could be clearly separate from each other and has various internal structures. Homogeneous compact structure, layered structure and clustered structures were proved to exist. For both flocs and granules, the mechanism of cell aggregation is initiated by environmental stress which triggers cell to cell interaction. Cell to cell interaction is a combined process which encompasses different physico-chemical and microbial forces. Physico-chemical forces are mostly derived from colloidal and surface chemistry such as the DLVO theory and different chemical bounds such as divalent cation bridging and hydrophobic interaction. Biological forces, such as EPS production and quorum sensing could either enhance the physico-chemical forces or act as main force. In the proposed flocculation mechanism, the changing environment is a prerequisite to initiate the cell movement, the microcolony and floc formation are the results of the change of cell surface characteristics which was triggered by QS. Different mechanisms of granulation were proposed by various authors. The previous view on the hydrodynamic selection at the cost of high biomass washout as main factor has been recently challenged by

different studies in which granules have been cultivated at low shear as well as at steady state operation, however, a longer cultivation time is required. This opens opportunities for engineers in the choice between a shorter granulation period and a longer SRT.

The studies on EPS have followed different approaches. For floccular sludge, emphasis was focused on the establishment of different EPS layers with performance on flocculation, settlelability and dewaterability. Quantification of different EPS compounds has also been applied in granular EPS study. Correlation between EPS quantity and granular performance/stability has been established. The results have always been contradictory. One of the reasons could be the use of non standardized extraction method. Another reason is that for EPS from both flocs and granules, their composition and distribution are process dependent. The new classification was proposed by Wang et al. (2014a) who distinguished EPS at cell level and microcolony level. According to the fractal structure of flocs (Fig. 2), the basic microcolony unit has a compact structure which is highly resistant to shear. It is reasonable to propose that at some condition the formation of such microcolony serves as precursor of granulation. The path towards the formation of bigger granules or towards coagulation into flocs may be dependent on other factors such as process health, hydrodynamic shear, substrate loading rate and microbial species. This provides insight in further studies on different mechanisms involved in microcolony formation and floc formation/stability. The spatial distribution of EPS in granules could be visualized by CLSM after selective staining. Enzymatic degradation of different components gives information on its respective correlation with granular stability. Study of granules and EPS as hydrogel is a novel approach which gives direct information on the strength of the granule and shed light into the molecular structure of EPS components. To date, other than the alginate-like structure, only one polysaccharide structure which is believed to be producedas gel has been elucidated (Seviour et al. 2010b). Further questions are: why, how and under which circumstances is this specific molecule synthesized? Is this molecule found only in one specific case or is it prerequisite material for general granulation process? More researches are of interest in this area and a general structure or compounds with similar specific chemical structure are expected to be discovered.

Activated sludge systems have been intensively applied in the form of flocsfor the past hundred years whereas granulation technology especially aerobic granulation started from late 1990s (Adav et al. 2008b). It could be concluded through the existing lab-scale and full-scale

aerobic granular systems that granulation from conventional flocculated activated sludge is both biologically and thermodynamically feasible (Liu et al. 2009). Experiences from activated sludge drive to assume that aerobic granulation is neither spontaneous nor random at optimum operation status for activated sludge system. As discussed in Chapter "Mechanisms of bioaggregates formation", there exist multiple forces which contribute to the granulation thus it could be hypothesized that more than one factor could serve as a trigger. Further studies especially on genetic background of bioaggregation are expected to fill the research gaps and facilitate its large scale application in wastewater treatment.

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Chapter 3

Evolution of nitrogen components and microbial diversity of Anammox enrichment process from different seeding sludge

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Chapter 3 Evolution of nitrogen components and microbial diversity of Anammox enrichment process from different seeding sludge

Summary

Autotrophic nitrogen removal which combines partial nitritation and anaerobic ammonium oxidation (Anammox) is an attractive technology suitable for high ammonium strength wastewater with low organic carbon content. However, the extreme slow growth of the Anammox bacteria with doubling time of 9-13 days hinders its wide full scale application. The aim of this research was to investigate the feasibility and operational strategy of Anammox enrichment from conventional aerobic sludge, denitrification sludge and anaerobic sludge using sequencing batch reactor (SBR) in two series of tests. In test 1, with a high selection stress and insufficient oxygen control, the reactor seeded with aerobic sludge reached 50-60% total nitrogen removal after 240 days whereas that seeded with anaerobic sludge failed to establish Anammox activity. In test 2, Anammox process was successfully established in the reactor seeded with denitrification sludge with a total nitrogen removal of approximately 80% after 150 days under strict oxygen control and low selection stress. Under the same operational condition, the reactor seeded with anaerobic sludge reached only 20-30% total nitrogen removal. All the reactors experienced fluctuating performances during the enrichment process, which was believed to be the consequence of inhibitory factors such as dissolved oxygen, free nitrite and free ammonia as well as undesirable coexisting bacteria which compete for the same substrate. The denaturing gradient gel electrophoresis (DGGE) band from the amplified DNA samples extracted from different enrichment stage showed a clear evolution of the microbial composition as reflected by the change in the band locations and their intensity.

Key words: nitrogen; Anammox; ammonium; nitrite

3.1 Introduction

The discovery of the Haber-Bosch process enabled the intensive use of nitrogen based fertilizers in agriculture and ultimately contribute as a main anthropogenic source of reactive nitrogen released in water body (Cherkasov et al. 2015). An excessively high concentration of nitrogen is toxic to both ecosystem and human health. For example, a high level of nitrate may also cause eutrophication in aquatic system. Free ammonia are toxic to aquatic organisms at concentration as low as 0.25 mg/L and nitrites are toxic to human which causes methemoglobinemia (Knobeloch et al. 2000). Thus the removal of reactive nitrogen compound, i.e. ammonium (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) from wastewater stream in commercial wastewater treatment plant (WWTP) is mandatory. The conventional treatment of nitrogen involves a complete nitrification step, in which ammonium is converted to nitrate under high dissolved oxygen (DO) condition by autotrophs, and a denitrification step, in which nitrate is reduced to non reactive nitrogen gas (N_2) under anoxic condition with the presence of organic carbon for heterotrophs **(Fig. 3.1)**. This process has achieved the most widely full-scale application for wastewater with relatively low nitrogen concentration i.e. total nitrogen concentration < 100 mg N/L, such as municipal wastewaters (Van Hulle et al. 2010). However, the use of the conventional process is limited when the wastewater contains high concentration of NH_4^+. Besides, high intensity aeration in the nitrification step and external carbon supply in the denitrification step are normally necessary, which contributes to a higher operation cost for the WWTP (Nozhevnikova et al. 2012).

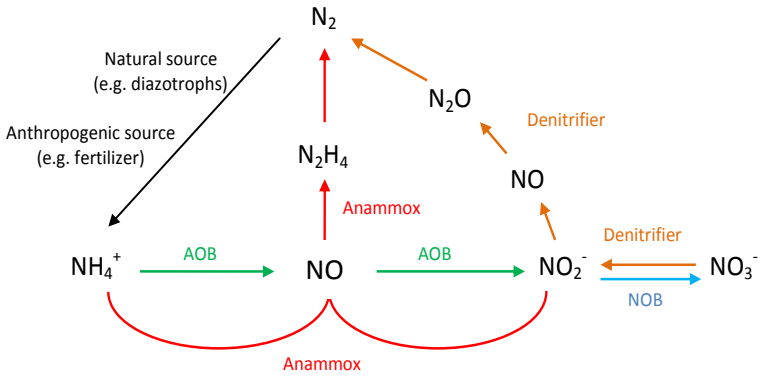


Fig. 3.1 Nitrogen cycle

The discovery of anaerobic Ammonium oxidation (Anammox) process offered an alternative to treat high ammonium strength wastewater with low carbon to nitrogen (C/N) ratio. The Anammox process was first discovered by Mulder et al. (1995) in a denitrifying fluidized bed

reactor and was proven by nitrogen and redox balance in continuous-flow experiments. The autotrophic Anammox bacteria are able to convert NH_4^+ to N_2 with nitrite as electron acceptor under anaerobic condition. The combined process of partial nitritation (Eq.1) and Anammox (Eq.2), which is generally called autotrophic nitrogen removal, has drawn wide attention in treating ammonium rich wastewater stream. The principle of the process was proposed by vandeGraaf et al. (1997):

$$NH_4^+ + 1.5O_2 + 2HCO_3^- + 2H_2^- + 3H_2O + 2CO_2 \quad \text{(Eq. 1)}$$

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.066_2 + 2.03H_2O + 0.066CH_2O_{1.5}N_{0.15} + 0.26NO_3^- \quad \text{(Eq. 2)}$$

Eq. 1 and Eq. 2 have been applied as intrinsic stoichiometry of Anammox process since 1999 in almost all of the studies worldwide. However, Eq.2 was updated by Lotti et al. (2014) (Eq. 3) through the operation of high purity free cell Anammox bacteria and data reconciliation which has high reliability.

$$NH_4^+ + 1.146NO_2^- + 0.071HCO_3^- + 0.057H^+ + 0.057H_2 + 2.002H_2O + 0.071CH_{1.74}O_{0.31}N_{0.20} + 0.161NO_3^- \quad \text{(Eq. 3)}$$

The most important advantage of this process lies in saving oxygen for nitrification and biodegradable organic carbon source for heterotrophic denitrifiers. Since the feed of oxygen is mostly achieved through aeration, which is an energy intensive process, significant energy saving can also be expected. **Table 3.1** compares the need of oxygen and biodegradable COD from different process on a stoichiometric basis. From the updated stoichiometry (Eq.3), more saving on oxygen demand for partial nitritation and even lower residual nitrate in the effluent could even be expected. Other than that, a significant decrease in sludge production has been achieved during the treatment of urban wastewater, primarily due to the slow growth rate of the autotrophic organisms (Strous et al. 1999). This could also result in a smaller size and simple configuration of the treatment plant, which is compliant with the concept of process intensification. Moreover, since nitrous oxide (N_2O) is not a by-product of Anammox process, the emission of greenhouse gas (GHG, such as CO_2, CH_4, N_2O etc.) is significantly lower than conventional treatments. Above all, this process results in a lower environmental footprint than the conventional nitrification/denitrification system in terms of oxygen and biodegradable COD consumption as well as a smaller treatment plant size.

Despite all the benefits observed, the application of Anammox process in wastewater treatment is quite limited at both pilot and full-scale. The main obstacle is the long start up time due to its extremely slow growth. The enrichment of Anammox biomass takes minimum 56 days (Zhou and Yao 2010) up to as long as 360 days (Shen et al. 2012). Enrichments from different conventional seeding sludge are found to be feasible (Chamchoi and Nitisoravut 2007; Qing et al. 2009; Shen et al. 2012; Wang et al. 2011). The first full scale Anammox WWTP took more than three years to scale up from a 10 L lab scale reactor to a 70 m^3 full scale reactor (van der Star et al. 2007). Furthermore, the process is sensitive to various inhibitory factors (e.g. dissolved oxygen, free ammonium, nitrite) (Jin et al. 2012). Thus a high monitoring and maintenance effort is required. The aim of this research was to investigate the feasibility and operational strategy of Anammox enrichment from conventional aerobic sludge, denitrification sludge and anaerobic sludge using SBR. The reactor performance was monitored by chemical analysis on influent/effluent chemical composition. The evolution of the microbial diversity was confirmed by microbial analysis.

Table 3.1 Comparison of different nitrogen removal process (Adapted from Van Hulle et al. 2010).

Process	**gO_2/gNH_4-N removed**	**$gCOD/gNH_4$-N removed**
Nitrification/denitrification	4.57	4.0
Nitritation/denitritation	3.43	2.4
Partial nitritation/Anammox	1.72*/1.49**	0

* Eq.2 is applied

** Eq.3 is applied

3.2 Materials and methods

Seedings ludge

Seeding sludge was selected from three full-scale reactors performing different wastewater treatment process. Aerobic and denitrification sludge was sampled from the aeration tank and denitrification tank, respectively, of a municipal WWTP located in Nola (NA), in southern

Italy. Anaerobic sludge was sampled from an anaerobic digester treating buffalo manure and milk serum located in Albanella (SA), southern Italy.

Feeding medium

Synthetic wastewater was prepared according to (deGraaf et al. 1996). The synthetic wastewater is composed of: $KHCO_3$, 1250 mg/L; KH_2PO_4, 67.5 mg/L; $MgSO_4.7H_2O$, 300 mg/L; $CaCl_2.2H_2O$, 180 mg/L; trace solution S1: 1mL/L; trace solution S2: 1 mL/L.S1 and S2 are concentrated trace solutions. S1 is composed of 5g/L $FeSO_4$ and EDTA. S2 is composed of different metals which are essential micro nutrients for bacteria (deGraaf et al. 1996). The present amount of $KHCO_3$ is sufficient to buffer the pH at around 7.5-8.0 and provide inorganic carbon for the autotrophic bacteria. $(NH_4)_2SO_4$ and $NaNO_2$ was added to the medium as nutrients with varying amounts/concentrations.

Reactor configuration and operation

Two identical 5L cylindrical glass SBRs with 4L working volume and H/D ratio of 4:1 were set up to perform the Anammox biomass enrichment **(Fig. 3.2)**. Two sets of tests have been conducted in sequence. In Test 1, reactor ASR and ANR1 were initially set up to compare the enrichment performance between aerobic sludge and anaerobic sludge, respectively. Both reactors were discontinuously flushed with non reactive nitrogen gas and sealed with Parafilm® manually to maintain anoxic condition. A temperature of 34 ± 1 ℃ was constantly maintained inside the reactors by an external thermostatic bath. The hydraulic retention time (HRT) was fixed to 2 days. The cyclic exchanging volume was set to 1 L. The working sequence lasted 12 hours and was composed of 4 phases as shown in **Table 3.2**. A system of magnetic stirring was applied to maintain the biomass suspended with minimum velocity during the biological reaction phase of the SBR cycle.

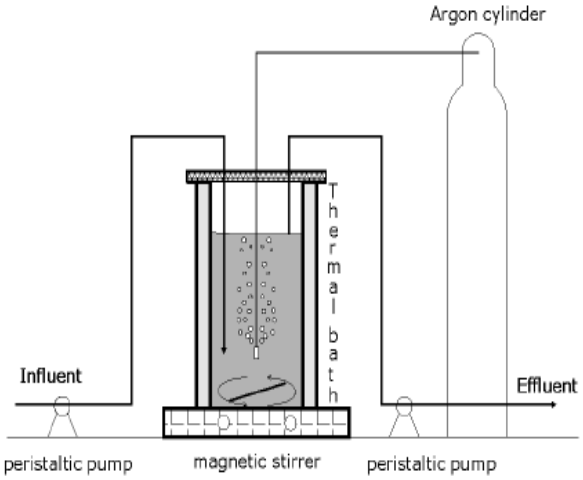


Fig. 3.2 Experimental set up of SBR

After the 240 days of test, ASR was emptied and replaced by denitrification sludge (DSR) and ANR1 was replaced with the same anaerobic sludge as previous test 1 (ANR2). Aiming at improving the operation of test 1, test 2 was conducted with stricter oxygen control and a lower selection stress. Anoxic conditions inside the reactors were maintained by closing them hermetically and regularly flushed by Argon gas. A temperature of 34 ± 1 ℃ ℃ was constantly maintained inside the reactors by an external thermostatic bath. Before feeding, the synthetic wastewater was appropriately degassed by a vacuum pump (KNF Laboport, N8xx series). HRT was set to 4 days with the same working volume and volumetric exchange ratio as the test 1. The working sequence lasted 24 hours and it was composed of 4 phases as shown in **Table 3.2**. The same magnetic stirring system was used to maintain the biomass suspended.

Feeding and discharging were performed by peristaltic pumps (Watson Marlow, mod. 520 Du). The on/off operations of all devices used in the experiments were regulated by an electronic timer (GIB). A summary of the reactor operations is presented in **Table 3.2**.

Table 3.2 Conditions and feeding strategies for the studied reactors.

	Reactor	**Volume, HRT, exchange volume ratio**	**Seeding sludge, initial VSS**	**O_2 control**	**Feeding strategy in each 12/24-hour cycle**
Test 1	ASR	4 L, 2 days, 25%	Activated sludge, 1.5 gVSS/L	Opening of the reactor closed by Parafilm®, reactor discontinuously	3 hours feeding and mix;

	ANR1	4 L, 4 days, 25%	Anaerobic sludge, 1.5 gVSS/L	flushed with nitrogen gas	8 hours mix and react; 45 min settling; 15 min discharging
Test 2	DSR	4 L, 2 days, 25%	Denitrification sludge, 3 gVSS/L	Opening of the reactor sealed hermetically by silicon; reactor regularly flushed by Argon gas; feeding medium degassed and flushed with Argon gas prior to feed	80 min feeding and mix; 16 hours and 40 min mix and react; 4 hours settling; 20 min discharging

Analytical methods

Effluent samples were collected every 2-4 days for chemical analysis to monitor the effluent composition.The ammonium (mg NH_4^+-N /L) concentration was measured by Nessler method using a spectrophotomer (PhotoLab 6600 UV-VIS series) as well as by distillation equipment (UDK 132 Semiautomatic Distillation Unit, VelpScientifica) when concentration was higher than 3 mgNH_4^+-N/L (Eaton et al. 2005). Nitrite and nitrate (mg NO_2^--N/L, mg NO_3^--N/L) were measured by ionic chromatography (761 Compact IC, Metrohm) and spectrophotometric equipment (PhotoLab 6600 UV-VIS series) (Eaton et al. 2005). The pH was measured by a portable pH meter (WTW, inolab). Total suspended solids (TSS) and VSS were determined according to standard method (Eaton et al. 2005).

Microbial analysis

Microbial analyses were performed on samples from reactor ASR, collected on 0, 30^{th}, 60^{th}, 120^{th}, 150^{th}, 210^{th} and 240^{th} days, and from reactor DSR, collected immediately after filling of reactor and at the end of experimentation (on day 150). Biomass was recovered from each sample and total genomic DNA was extracted using the Fast DNA Spin Kit for Soil (MP Biomedicals) according to the supplier's recommendation. The primers V3f (5'-CCTACGGGAGGCAGCAG-3') with a GC-clamp according to Muyzer et al. (1993) and V3r (5'-ATTACC GCGGCTGCTGG -3'), spanning the V3 region of the 16S rDNA, were used for polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE) analysis of bacterial populations. The PCR mixture was prepared as reported by Pepe et al. (2013). The PCR conditions were performed as described by Palomba et al. (2011). PCR

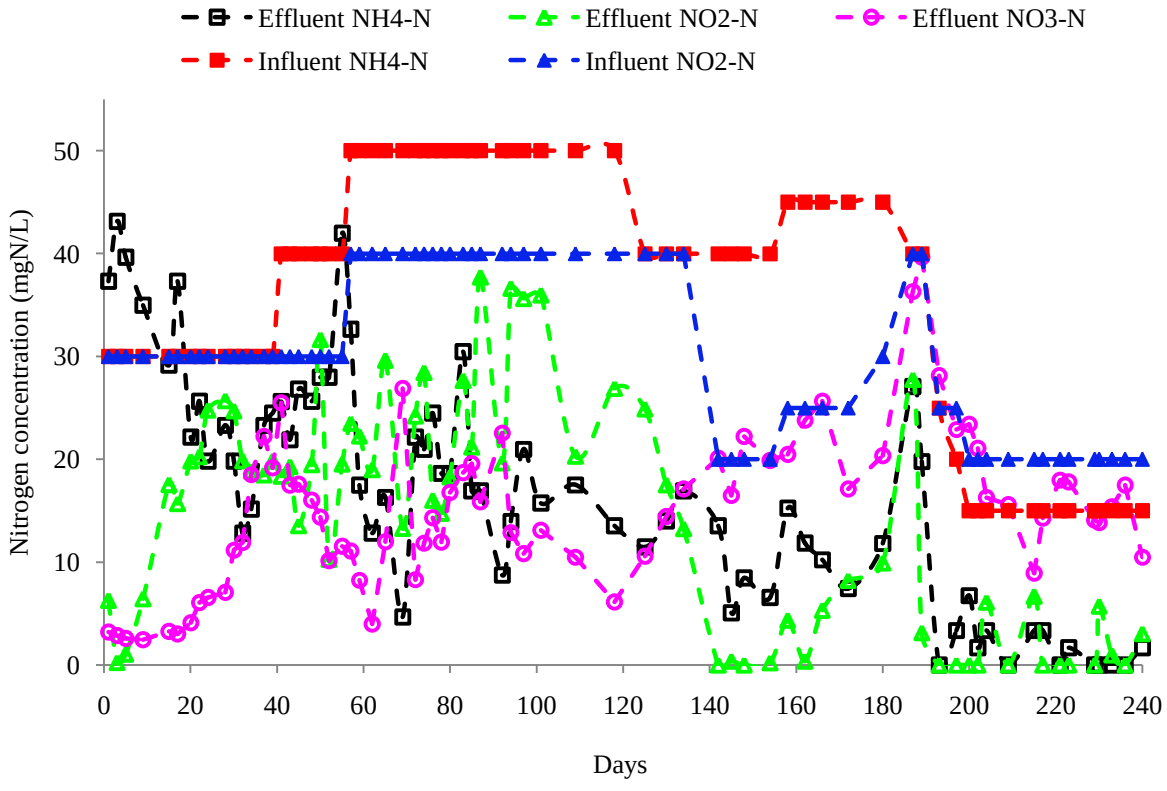
products were analyzed by DGGE in a 0.8 mm polyacrylamide gel using a Bio-Rad Code Universal Mutation System (Bio-Rad, Laboratories, Milan, Italy) as described by Ventorino et al. (2013). Statistical analyses were performed automatically using the software Phoretix 1 advanced version 3.01 (Phoretix International Limited, Newcastel upon Tyne, England). The correlation matrix of DGGE patterns, obtained using the method described by Saitou and Nei (1987), was used for cluster analysis using Systat 5.2.1 as previously described by Ventorino et al. (2007).

3.3 Results

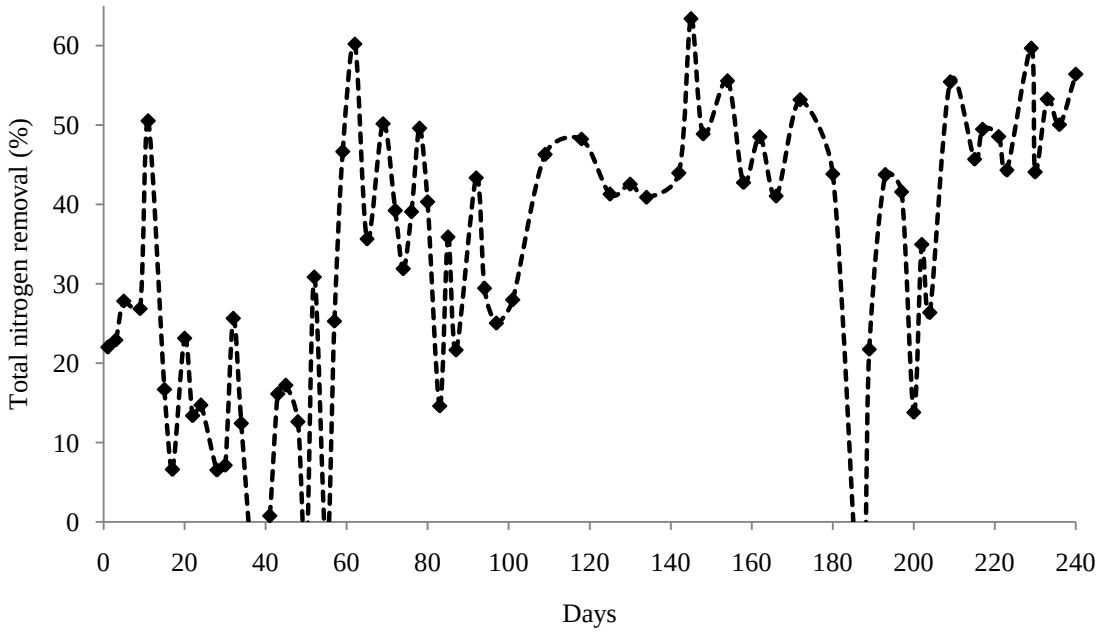
3.3.1 Anammox enrichment from activated sludge

ASR and DSR were inoculated with activated sludge from the aeration and denitrification reactor, respectively. In test 1, the nitrogen profile of ASR showed clear "three phases" (Fig 3.3a), which is similar to other studies (Chamchoi and Nitisoravut 2007; Huang et al. 2014; Wang et al. 2011). From day 0 to day 20, the reactor was dominated by denitrifiers which converted most of the NO_2^- and NO_3^- to nitrogen gas. Since no organic carbon source is supplied, denitrification activity soon diminished when organics from cell lysis were completely consumed. The accumulation of NH_4^+ was supposed to be caused by the lack of oxygen for ammonium oxidation bacteria (AOB) as well as the hydrolysis of nitrogen compound released from cell lysis. This is so called "denitrification dominant period". The "Anammox propagation phase" lasted from day 20 to day 140 where nitrite accumulation remained the main problem. At the mean time, due to lack of control of dissolved oxygen, NO_3^- always presented at high level for the whole period regardless of the feeding load. NH_4^+ conversion was low in the first 40 days of operation, presumably due to the few amount of Anammox and inhibition of AOB. The highest total nitrogen removal of about 60% was reached on day 60 (Fig. 3.3b), which leads to the decision of further increase of the total nitrogen load to 45 mgN/L.d from day 60 to 120. The simultaneous occurrence of NO_2^- and NO_3^- and low total nitrogen removal of less than 15% on day 83 might imply the presence of both AOB and NOB. The removal was restored to 48% on day 118 possibly due to adaptation. The increase of total nitrogen load from day 40 and further increase from day 60 resulted in more serious NO_2^- accumulation than NO_3^-. With the aim of bringing the NO_2^--N/NH_4^+-N ratio close to the stoichiometric 1.146 without overloading the reactor, the total nitrogen

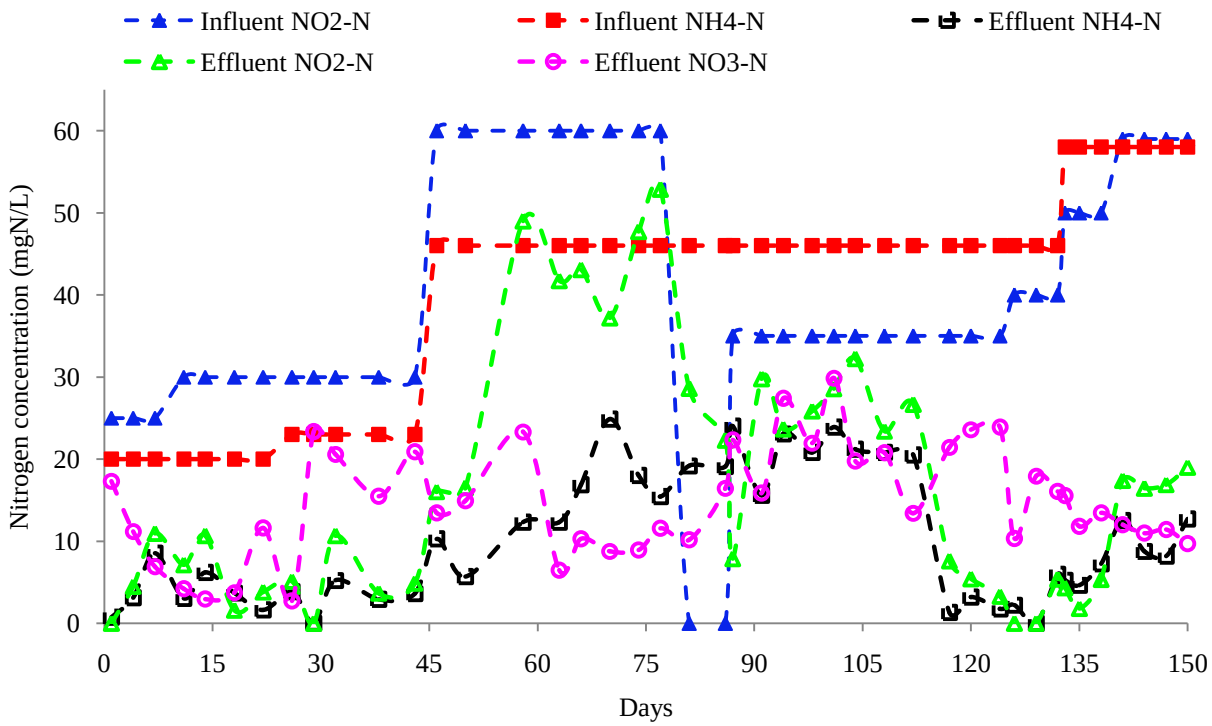
loading was lowered to 40 mgN/L.d with equal NH_4^+-N and NO_2^--N concentration from day 125 to 134. As a consequence, the nitrite concentration decreased quickly while nitrate kept increasing. The high effluent nitrate concentration may indicate the thriving of nitrite oxidizing bacteria (NOB) which competes for NO_2^- with Anammox. A reduction of influent NO_2^- from day 142 to 154 did not remediate this problem while resulted in a higher conversion of NH_4^+. The increase of both substrates in the next 15 days resulted in further NO_3^- accumulation, an increase of NO_2^- and a decrease of NH_4^+ in the effluent with total nitrogen removal of about 40-50%. This indicates that the presence of AOB and NOB may play a protective role against oxygen for Anammox (Vlaeminck et al. 2009). However, further increase of the total nitrogen load resulted in a loss of nitrogen removal on day 187. The Anammox process was recovered after reducing the load to17.5 mgN/L.d with the NO_2^--N/NH_4^+-N ratio of 1.32 (Eq. 2). The total nitrogen removal stabilized at about 50-60% with a high nitrate level (Fig. 3.3b).



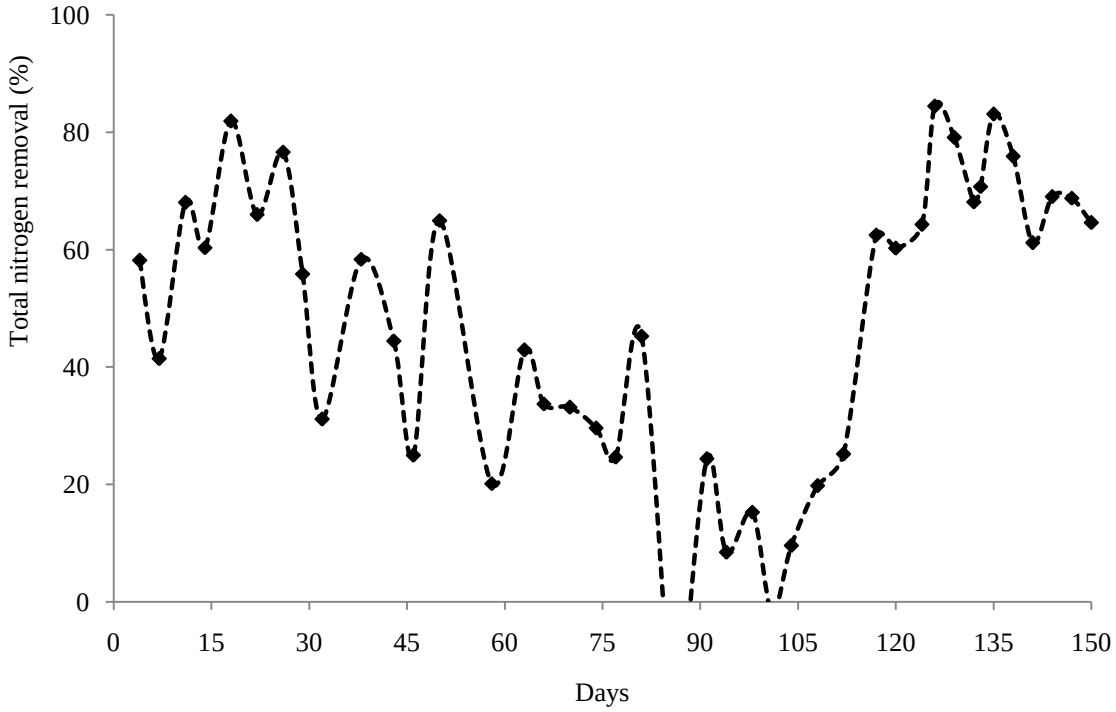
(a)


Total nitrogen removal (%)
0
10
20
30
40
50
60
0
20
40
60
80
100
120
140
160
180
200
220
240
Days

(b)


Influent NO2-N
Influent NH4-N
Effluent NH4-N
Effluent NO2-N
Effluent NO3-N
Nitrogen concentration (mgN/L)
0
10
20
30
40
50
60
0
15
30
45
60
75
90
105
120
135
150
Days

(c)



(d)

Fig. 3.3(a) Evolution of nitrogen composition in ASR; (b) Evolution of total nitrogen removal in ASR; (c) Evolution of nitrogen composition in DSR; (d) Evolution of total nitrogen removal in DSR

Reactor DSR was inoculated with sludge from the denitrification tank of the municipal WWTP. When an HRT of more than 4 days was applied, the reactor experienced high $NH_4^+$and NO_2^- accumulation and an extra longer denitrification domination phase. Therefore, a low nitrogen load was controlled to 25 mgNO_2^--N/L.d and 20 mgNH_4^+-N /L.d until a simultaneous removal of NH_4^+ and NO_2^- was observed (designated as day 0 in Fig. 3.3c). During this period, oxygen was strictly controlled by (i) flushing the influent with Argon gas; (ii) hermetically sealing the reactor and (iii) regularly flushing the reactor with Argon gas.

When simultaneous removal of NH_4^+ and NO_2^- has been observed, the influent NO_2^--N/NH_4^{+-}-N was kept at the previous stoichiometric value of 1.32 (Eq. 2) but with total nitrogen load of 11.25 mgN/L.d. A total nitrogen removal of 70% could be achieved (Fig. 3.3d) at this low substrate load. However a low NO_3^--N/NH_4^+-N and high NO_2^--N/NH_4^{+-}-N ratio in the effluent (Fig. 3.4) might indicate the presence of remaining denitrifiers which could live on the

organics released from cell lysis. Thus from **day 8-26**, the NO_2^-/NH_4^+ value was increased to 1.5 to allow sufficient nitrite for Anammox. A highest removal was achieved on day 26 with a total removal of 76%. However the total nitrogen loading was low and the reactor performance was very unstable and serious NO_2^- accumulation occurred after day 20. Also a high NO_3^- concentration of 11.6 mgN/L was observed on day 22 which was much higher than the stoichiometric level. The high concentration of oxidized nitrogen in effluent indicated the complete wash out of denitrifiers due to the lack of organic carbon source. The low effluent ammonium level also implies the existence of AOB and NOB and the possible oxygen leakage. From **day 26-45**, the influent NO_2^--N/NH_4^{+-}-N was reset to 1.32 by increasing the NH_4^+ level to 23 mgN/L. A total nitrogen removal decreased significantly during this period with an average nitrate level as high as 20 mgN/L. This leaded to the decision of recheck the closure of the reactor and controlling the dissolved oxygen inside the reactor by a DO meter. The high nitrate level implies that NOB could survive at DO level as low as 0.2 mgO_2/L and could be reactivated as soon as DO is elevated. Thus oxygen control is crucial in Anammox enrichment when the seeding sludge contains NOB. From **day 46-78**, the influent NH_4^+ and NO_2^- were doubled with the aim of stimulating the growth of Anammox and keep free NH_3 and HNO_2 level at its optimum level. However, a good total nitrogen removal of 60-70% only occurred until **day 50**, followed by a sharp decrease to 13.5% on day 78. The loss of total nitrogen removal coincided with serious nitrite accumulation which resulted in nitrite concentration as high as 50 mgN/L in the effluent. This may imply the toxicity of a high level of NO_2^- for Anammox bacteria. However, NO_3^- kept an average low level of 10 mgN/L during this period, indicating the deactivation of undesired NOB and effective control of oxygen. To remediate this situation, only NH_4^+ was added in the feeding for the next seven days (**day 79-85**) which lead to an effective decrease of NO_2^-, presumably due to the consumption of Anammox bacteria as well as a dilution effect. As a consequence, the calculated total nitrogen removal (%), which is calculated based on the total nitrogen concentration (mgN/L) of influent and effluent, is below zero. To prevent further accumulation of nitrite, the NO_2^--N/NH_4^{+-}-N ratio was kept 0.76 from **day 87-125**. Total nitrogen removal was re-established to 71% until day 125 with fluctuating performance and frequent nitrate accumulation. A significant NH_4^+ removal was observed from day 117 indicating good performance of the Anammox process. From **day 126-132**, the NO_2^--N/NH_4^{+-}-N ratio increased to 0.87 by increasing the influent NO_2^- concentration to 40 mgN/L with the aim of gradually bringing the NO_2^--N/NH_4^{+-}-N ratio to its stoichiometric level without causing accumulation. Total nitrogen removal maintained at 70-80% without accumulation of NO_2^- and NO_3^-. To further promote

the good performance at this condition, the total nitrogen load increased to 27 mgN/L.d from **day 133-138** with NO_2^--N/NH_4^{+-}-N equal to 0.86. The total nitrogen removal remained 70-90% while the ratio of NH_4^+:NO_2^-:NO_3^- was found closer to its stoichiometric value (both Eq. 2 and Eq. 3) with a bit higher NO_3^- content (Fig. 3.4). This good performance proves the establishment and domination of Anammox activity inside the reactor. From **day 139-150**, NO_2^--N/NH_4^{+-}-N was brought to 1.02 with a total nitrogen load of 30 mgN/L.d. The total nitrogen removal remained about 70% and a ratio of NH_4^+-N:NO_2^--N:NO_3^--N close to its stoichiometric value (Eq. 3) was achieved. Thus the stable phase of the Anammox process was considered reached. Alkalinity and pH of the influent and effluent were measured throughout the enrichment period. The presence of excess inorganic carbon as compared to the stoichiometric amount according to Eq. 1 sufficiently buffered the reactor against strong pH variation (7.5-8.0). A higher amount of alkalinity was consumed before day 134 (data not shown). This might indicate that AOB or NOB were still active inside the reactor because nitrification requires much more inorganic carbon than Anammox (Eq. 1).

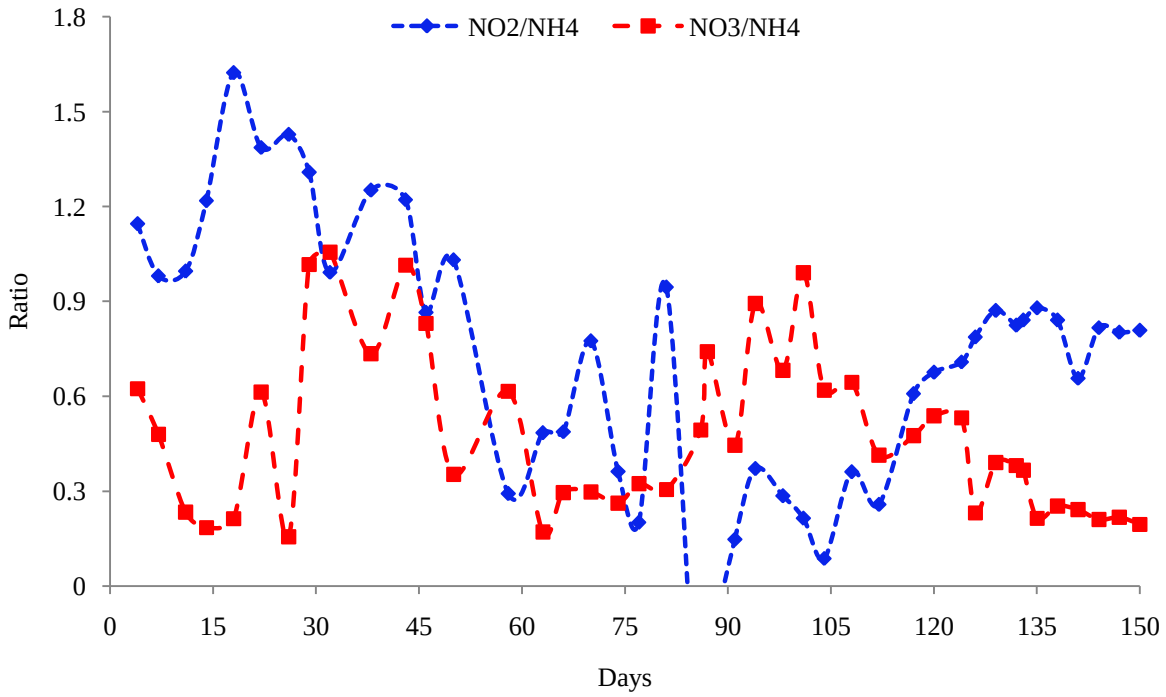


Fig. 3.4 ratio of NO_2^- removed to NH_4^+ removed and NO_3^- produced to NH_4^+ removed in DSR

Compared to other studies (Chamchoi and Nitisoravut 2007; Huang et al. 2014; Wang et al. 2011), both of our tests on activated sludge experienced more fluctuating performance in

response to trivial change of the operation conditions. This further proves that Anammox bacteria is sensitive to environmental parameters such as dissolved oxygen, free ammonium and nitrite concentration as well as the presence of other bacteria that compete for the same substrates. The control of these parameters is of crucial importance during the whole enrichment process.

3.3.2 Anammox enrichment from anaerobic sludge

Anammox enrichments from various anaerobic sludge have been reported in literature (Chamchoi and Nitisoravut 2007; Huang et al. 2014; Wang et al. 2011). However, the enrichment from anaerobic floccular sludge treating buffalo manure was not successfully established after two attempts. To study the effect of seeding sludge and operation condition, reactor ANR1 was operated in parallel with reactor ASR whereas reactor ANR2 was operated in parallel with DSR. With a shorter HRT of 2 days and no strict control of dissolved oxygen, ANR1 did not show any evidence of simultaneous nitrogen removal after 120 days thus was considered failed and discarded.

Up till day 44, ANR2 showed similar pattern as DSR at low total nitrogen load of lower than 26.5 mgN/L.d, which meant similar microbial shift occurred inside the reactor (Fig. 3.5). From day 46 to 79, the same nitrite accumulation was observed with a slightly higher NH_4^+ conversion (about 85% for ANR2 vs. 75% in DSR) (Fig. 3.3c and Fig. 3.5), probably resulted from a higher AOB activity due to leakage of oxygen. From day 126, ANR2 did not show a reduction of NO_3^- and improvement of NH_4^+ and NO_2^- removal, which occurred in DSR. And the total nitrogen removal reduced to 10% on day 128. The final nitrogen removal stabilized at about 10-20% from day 130-150.

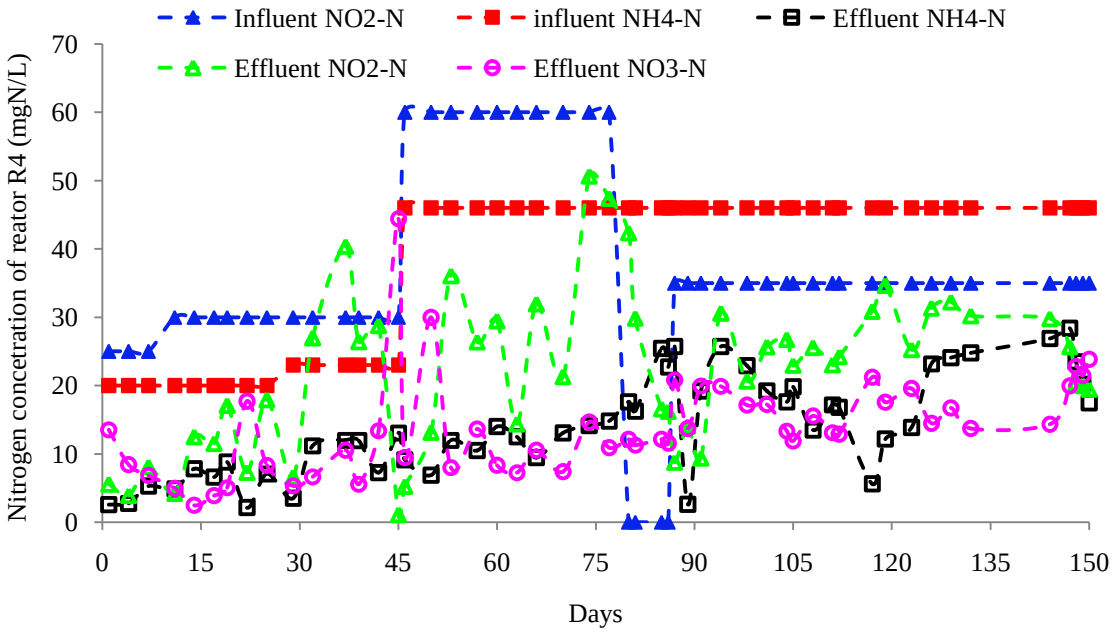


Fig. 3.5 Evolution of nitrogen composition in ANR2

3.3.3 Microbial analysis

Bacterial population dynamics was analyzed in the reactor ASR during the entire process using the culture-independent approach PCR-DGGE. This methodology allows the evaluation of the genetic diversity and the dynamics of complex bacterial communities in the environment based on different base-pair sequences of amplicons with the same length (Ge et al. 2015). The DGGE band patterns shown in Fig. 3.6a illustrate the prokaryotic communities observed throughout the entire Anammox enrichment process. The DGGE profiles of the bacterial populations show that the number of bands detected during the process in the reactor ASR remained similar until day 240 ranging from 32 to 40. Because the differences were mainly due to the different position and intensity of the bands rather than their number, a statistical analysis was performed. Cluster analysis (Fig. 3.6a) showed a high bacterial diversity and variation in the microbial community structure identifying two major groups (cluster 1: a-b; cluster 2: c-g) in which slight changes within the microbial populations were observed (similarity level from 85 to 100 %). The major cluster 1 included the initial phase of the process until day 30, while the cluster 2 grouped the samples from days 60 to 240. In particular cluster 1 showed a similarity level of 77% with cluster 2.

Microbial diversity in the reactor DSR was evaluated only immediately after the filling of the reactor and at the end of the process (day 150) (Fig. 3.6b). Although the band quantity was 27 and 25 for sample f and sample g, respectively, microbiota recovered after 150 days drastically changed (Fig. 3.6b). In fact, the similarity between these two samples was only 54% since variations in both the position, reflecting the diversity of microbial communities, and the intensity of the DGGE bands, indicating changes in relative abundance, were observed. This marked change in the bacterial community structure could be due to the growth and the activity of microbial populations involved in the Anammox process.

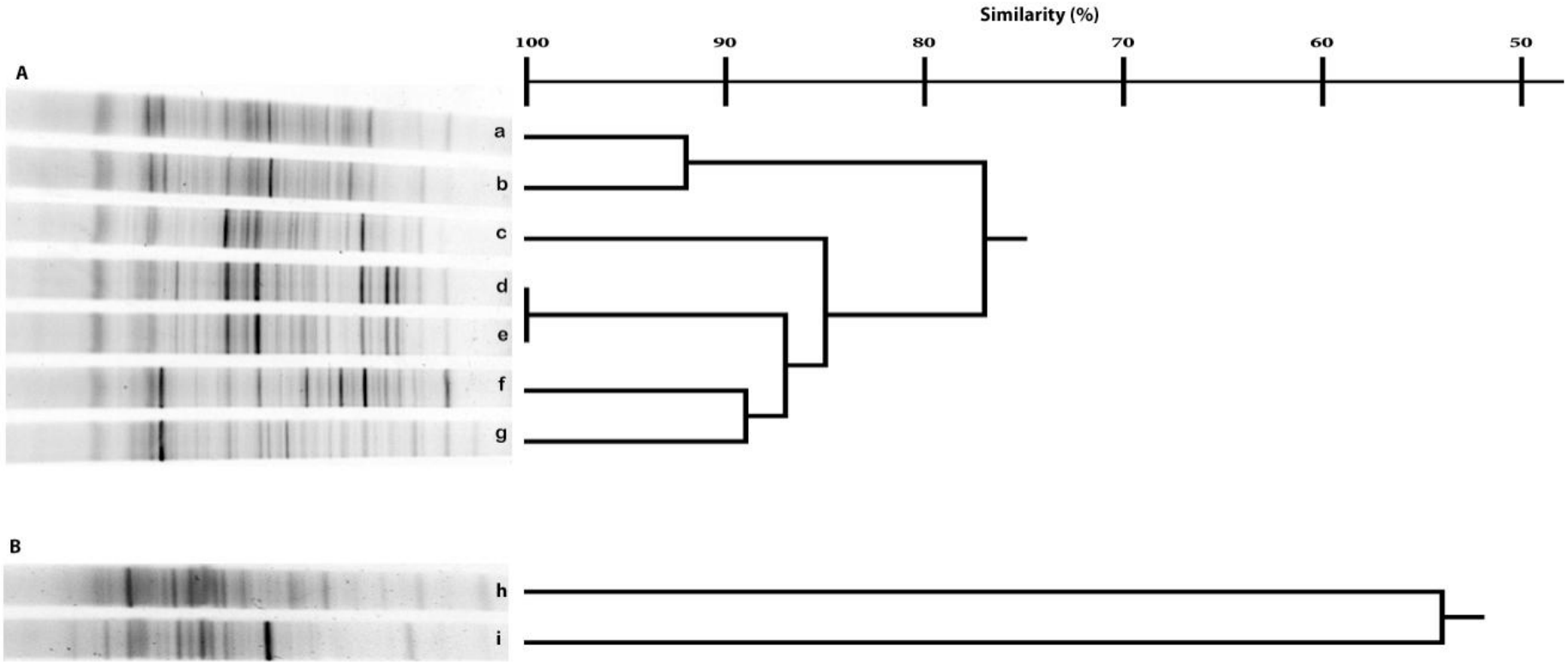


Fig. 3.6 DGGE profiles and dendrogramshowing the degree of similarity (%) of PCR-DGGE profiles of bacterialpopulations from reactor ASR during the Anammoxenrichmentprocess (panel A) and from reactor DSR immediatelyafterfillingreactor and at the end of the Anammoxenrichmentprocess (panel B). Lanes panel a: a, 0 d; b, 30 d; c, 60 d; d, 120 d; e, 150 d; f, 210 d; g, 240 d.Lanes panel b: h, 0 d;i, 150 d.

3.4 Discussion

3.4.1 Factors affecting the Anammox enrichment process

The extremely slow growth of the Anammox bacteria determines the operation strategy of low selection pressure (De Clippeleir et al. 2009). In the enrichment process, different selection pressures are suggested to be applied among different phases according to the evolution of microbial composition. During denitrification phase, a higher selection, which is achieved by a shorter HRT, is preferred to quickly washout the residual COD and denitrifiers which could potentially outcompete Anammox due to its higher affinity to NO_2^- and a much higher growth rate. With the depletion of COD, denitrifiers experienced an increasing decay and inhibited growth and finally washed out from the reactor. During the propagation phase, it was found that although under oxygen limited condition, AOB and NOB could quickly restore activity when oxygen is available from unexpected leakage. Thus while keeping a long HRT and SRT to minimize the selection stress, oxygen control is of crucial importance. However, a long SRT may result in growth independent metabolism which is also unfavorable for Anammox enrichment (Monballiu et al. 2013).

The amount of Anammox bacteria present in the seeding sludge was believed to be significant in shortening the required enrichment time (Wang et al. 2011). However, Kanders et al. (2014) found that Anammox bacteria present in the seeding sludge did not contribute to a shorter start up time while an appropriate boundary condition for Anammox growth was more important. Various conventional sludge as well as natural samples have been used as seeding sludge for Anammox enrichment, among which, activated sludge (Araujo et al. 2011; Dapena-Mora et al. 2004) and anaerobic granular sludge (Qing et al. 2009; Tran et al. 2006) are mostly preferred. In the present study, denitrification sludge as seeding sludge proved to be best suitable for Anammox enrichment presumably due to the anoxic condition of the WWTP. This is in accordance with the very first case where Anammox activity was discovered (Mulder et al. 1995). The aerobic sludge was taken from the aeration tank where DO is at sufficiently high level of 2-3 mgO_2/L. Thus it is less likely that Anammox bacteria could present in high amount and remain active. Therefore the seeding source at least partially led to a more difficult and fluctuate enrichment process and a lower final nitrogen removal. Despite that anaerobic granular sludge proved to be suitable for Anammox enrichment by

other authors (Qing et al. 2009; Tran et al. 2006), enrichment from anaerobic floccular sludge was not successful in this study. The reason for the low activity from anaerobic sludge despite a stricter oxygen control could be the lack of electron acceptor present in its original WWTP due to a strong reducing condition which is a characteristic of anaerobic process. Thus the environment was not favorable for the survival of Anammox.

The effect of dissolved oxygen (DO) was apparent from the excessively high production of NO_3^- in all reactors. Without control of oxygen in both influent and inside reactor, ASR experienced continuous NO_2^- and NO_3^- accumulation throughout the enrichment period under an average DO level of 1 mg/L. Anammox activity was established only under very low total nitrogen load of 17.5 mgN/L.d and removal efficiency of 50%. Due to its higher growth and higher affinity to nitrite, and taking into consideration that Anammox enrichment was established under long SRT, NOB can neither be excluded nor inhibited by the end of the enrichment process. In comparison, the oxygen in DSR was strictly controlled both in the influent and reactor. Jetten et al. (2001) reported an inhibitive DO value of 2 µM (0.064 mgO_2/L) for Anammox activity. In practical, the threshold value of 0.2 mgO_2/L was chosen (Jung et al. 2007). The DO was kept below 0.2 mgO_2/L by flushing the reactor with Argon gas as well as degassing of the influent prior to feeding. However, the NO_3^- produced/NH_4^+ removed ratio of DSR remained higher than the stoichiometric value (Eq. 2 and 3). This might imply that the control of oxygen is crucial in Anammox enrichment process and that NOB could survive even under very low DO (< 1 mgO_2/L).

The inhibitory effects of free NH_3 (FA), free nitric acid (FNA) and nitrite have been reported by different authors (Fernandez et al. 2012; Jaroszynski et al. 2012; Lotti et al. 2012). FA and FNA are pH dependent value calculated from Eq. 4 and Eq. 5 (Van Hulle et al. 2010):

$$[NH_3] = \frac{[TAN] \times 10^{pH}}{e^{-2300/(T+273)} + 10^{pH}} \qquad \text{(Eq. 4)}$$

$$[HNO_2] = \frac{[TNO_2] \times 10^{-pH}}{e^{-2300/(T+273)} + 10^{-pH}} \qquad \text{(Eq. 5)}$$

The lowest FA threshold of 1.7 $mgNH_3$-N/L was proposed by Jung et al. (2007) while a much higher value of 20 $mgNH_3$-N/L was found by Fernandez et al. (2012). Thus the inhibitory

threshold of FA is supposed to be case specific. In the present study, a high FA in reactor DSR from day 63-74 was associated with low total nitrogen removal as well as nitrite accumulation (Fig. 3.7). From day 87-112, FA was close to or higher than the minimum threshold value of 1.7 mgN/L, which was assumed to contribute to the deterioration of reactor performance. From day 117 in DSR, the reactor reached stable nitrogen removal while the FA level kept below 1.7mgN/L. For reactor ANR2, FA concentration constantly exceeded 2 mgN/L after day 85, which resulted in long term inhibition of Anammox activity. According to a pH dependent test of nitrite inhibition by Lotti et al. (2012), a higher correlation between Anammox activity and total nitrite concentration was found than the correlation between free nitric acid (FNA) and Anammox activity. Thus, total nitrite instead of FNA was proposed to be the main parameter involved in the inhibition. Due to the buffering of the bicarbonate in the influent, pH remained 7.5-8.5 throughout the enrichment period. Under this pH range, the free HNO_3 concentration was kept below the level of 6.6 $\mu gHNO_3$-N/L in DSR most of the time, which is the reported FNA inhibition threshold (Fernandez et al. 2012). Concentrations ranging from 5 to 100 $mgNO_2^-$-N/L of total nitrite have been reported to be inhibitive to Anammox activity (Jin et al. 2012). In the present work, high level of nitrite occurred during the nitrite accumulation period (day 58-77) in DSR. This coincided with a high FA level, both of which were at toxic level. The reactor performance restored in one month after NO_2^- concentration decreased to lower level. This is in accordance with Monballiu et al. (2013) who also found that inhibition by nitrite at such level was reversible. Similar observation could be found in ASR and ANR2. However in ANR2, the reactor performance was not restored by the end of the 150 days, which implies a long-term inhibition caused by a combination of factors including DO and FA.

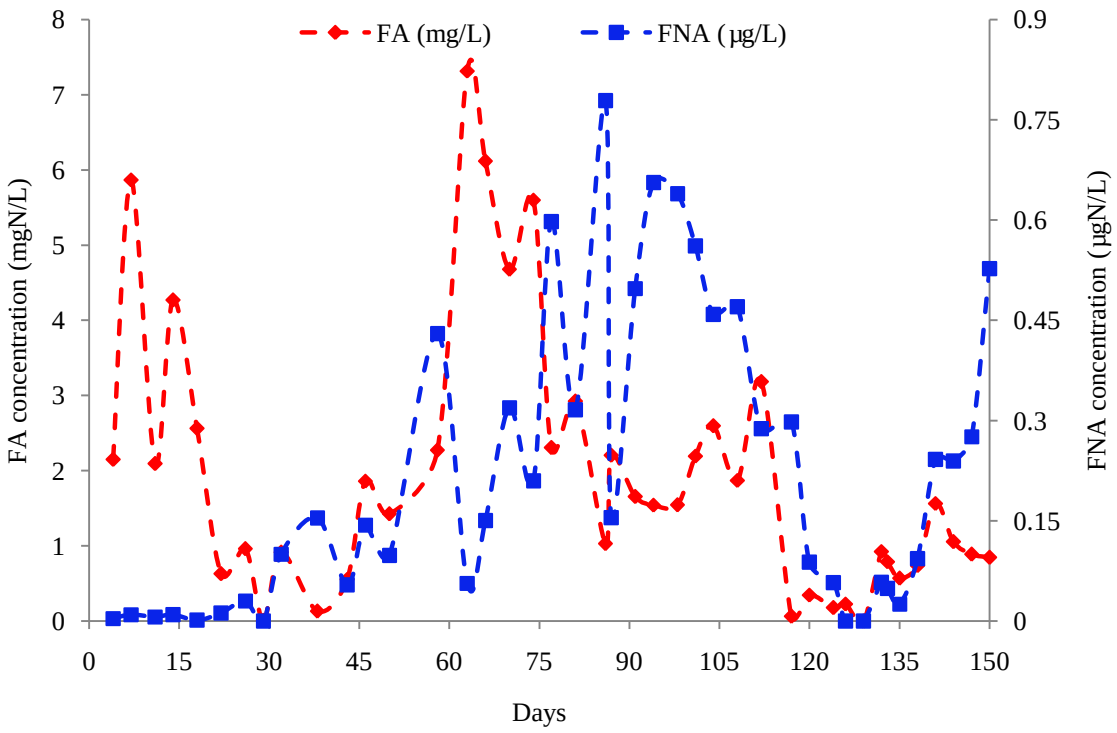


Fig. 3.7 Concentrations of FA and FNA in DSR

In conclusion, denitrification sludge proved to be most suitable as seeding sludge for Anammox enrichment under a strict oxygen control and low selection pressure. The highest total nitrogen removal rate reached 58 mgN/L.d with a removal of 70% after 150 days of operation. Reactors seeded with anaerobic sludge did not achieve a desirable nitrogen removal after two attempts, which implied that anaerobic sludge treating buffalo manure is not suitable for Anammox enrichment. A higher selection pressure is preferred during the "denitrification prevail phase" in order to quickly washout the denitrifiers and residual COD. AOB and NOB might be persistent in the reactor due to its higher growth rate and a long SRT required for the retention of Anammox bacteria. Thus oxygen control is of crucial importance in the enrichment process, not only because a high DO maybe inhibitive to Anammox but also the presence of oxygen could enable the thrive of AOB and NOB which compete with Anammox for substrate. A decrease of nitrogen removal is associated with an FA and nitrite concentration higher than its inhibitive threshold. The results from DSR and ASR also show that Anammox process can quickly be restored after being inhibited.

3.4.2 Microbiota dynamics

The change of the DGGE band patterns over the enrichment process correlated well with the process performance of the reactors. According to the process data from ASR (Fig. 3.3a and 3.3b), until day 20 the activity inside the reactor was dominated by denitrifiers and that the conversion of both NH_4^+ and NO_2^- remained low until day 40. This explains the high similarity of 92% among samples of cluster 1 (Fig. 3.6a), presumably corresponded to the presence of AOB and NOB. The change of DGGE profile on day 60 coincided with the fact that the highest nitrogen removal was observed on day 60 with stoichiometric value close to Eq.3. Since day 60 microbial populations in ASR significantly changed, it could be assumed that Anammox bacteria became one of the predominant groups. Despite fluctuation of nitrogen removal performance, bacterial community on day 60 was up to 85% similar to the microbiota recovered from day 120 to day 240. In addition, in the middle phase of the process the microbial community structure remained stable observing a similarity level of 100% between sample d (day 120) and e (day 150). The slight variation could be the result of nitrite accumulation from day 78 until day 140 which caused inhibition of Anammox and thriving of NOB. Towards the end of the enrichment process (day 210-240), sample f and g showed 89% similarity as reflected by the stabilized nitrogen removal. Variation of the band patterns through the presence or absence of prominent bands suggested the growth and the activity of different bacterial species.

In comparison to ASR, DSR experienced much higher variation in DGGE pattern from the seeding sludge to the end of the enrichment process (52% vs. 77%) (Fig. 3.6), presumably due to a much higher Anammox abundance. This is in well accordance with the process performance of DSR, in which Anammox process was successfully established with stoichiometric nitrogen compounds at a much higher total nitrogen load than ASR after 150 days. Thus it is assumed that Anammox bacteria compose the predominant group in DSR.

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Chapter 4

Evolution of extracellular polymeric substances (EPS) from Anammox biomass during enrichment in lab-scale bioreactor

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Ding, Z., Bourven, I., van Hullebusch, E., Panico, A., Pirozzi, F., Esposito, G., and Guibaud, G. Evolution of extracellular polymeric substances (EPS) from Anammox biomass during enrichment in lab-scale bioreactor

Chapter 4 Evolution of extracellular polymeric substances (EPS) from Anammox biomass during enrichment in lab-scale bioreactor

Summary

Extracellular polymeric substances (EPS) from different Anammox biomass have been extracted and characterized by quantitative and qualitative analysis to investigate its correlation with the enrichment process in a lab scale bioreactor. A decrease of protein to polysaccharide (PN/PS) ratio and an increase in total EPS extraction yield were observed during the enrichment process. The three dimensional excitation emission matrix (3D-EEM) showed similar location of the fluorescence peaks for all samples while samples with Anammox bacteria possess two distinct peaks in the low excitation wavelength range. Multi-excitation peaks may occur as evidenced by the identical fluorescence chromatogram after size exclusion chromatography (SEC) separation at excitation/emission 221/350 nm and 280/330 nm. Ultraviolet (UV) absorbance at 210 nm was recorded simultaneously with fluorescence detection at excitation/emission wavelength 222/300 nm, 221/350 nm and 280/330 nm after separated by SEC. With the enrichment of Anammox bacteria, UV chromatogram showed increase in both intensity and number of peaks, whereas fluorescence chromatograms showed similar peak number and only increase in intensity. An increase of hydrophobicity was observed during the enrichment process. The results of this study are expected to extend the knowledge of EPS evolution of Anammox enrichment process as well as provide novel approach for the characterization of EPS extracted from Anammox sludge.

Key words: Anammox; extracellular polymeric substances; protein; fluorescence; size exclusion chromatography; hydrophobicity

4.1 Introduction

The use of nitrogen fertilizer and increased human induced nitrogen pollution has made the nitrogen removal an important element in the wastewater treatment process. The conventional nitrification/denitrification treatment has achieved the most widely full-scale application for wastewater with low nitrogen concentration *i.e.* total nitrogen concentration < 100 mg N/L, such as municipal wastewaters (Van Hulle et al. 2010). Anaerobic Ammonium Oxidation (Anammox) was firstly observed by (Mulder et al. 1995). It is a process where ammonium is anaerobically oxidized into nitrogen gas with nitrite as electron acceptor mediated by autotrophic bacteria which belong to the group *Planctomycetes*. Coupled with partial nitrification, the Anammox process provides a "short-cut" option of ammonia removal from wastewater in comparison to the conventional nitrification/denitrification process. The process is summarized by the following reactions (Strous et al. 1999; Lotti et al. 2014):

$$NH_4^+ + 1.5O_2 + 2HCO_3^- \rightarrow NO_2^- + 3H_2O + 2CO_2 \quad \text{(Eq.1)}$$

$$NH_4^+ + 1.146NO_2^- + 0.071HCO_3^- + 0.057H^+ + 0.057H_2 + 2.002H_2O + 0.071CH_{1.74}O_{0.31}N_{0.20} + 0.161NO_3^- \quad \text{(Eq. 2)}$$

The most obvious benefit of the process is that, compared with the conventional nitrification/denitrification system, the nitrogen removal can be achieved with (i) the absence of biodegradable chemical oxygen demand (COD), (ii) lower oxygen supply and (iii) lower energy consumption. However the low growth rate (doubling time 11 days) and poor cultivability of Anammox bacteria have hindered its industrialized application (Jetten et al. 1998). The importance of *inocula* source has been reported towards the conclusion that *inocula* with high concentration of Anammox bacteria show better performance in enrichment especially in terms of shorter enrichment period and higher removal efficiency after steady state is reached (Wang et al. 2011).

Anammox bacteria tend to grow as aggregates, such as granules and attached growth biofilm, probably as a survival strategy due to its slow growth. The production of EPS is essential in the formation of floc, aggregates and biofilm (Ding et al. 2015). EPS are sticky highly hydrated matrices which result from microbial cells excretion under stress conditions, cell lysis as well as absorption of molecules from the bulk liquid. The composition of EPS mainly

includes polysaccharide, protein, humic-like substances, lipids, nucleic acids, uronic acids and inorganic components (Sheng et al. 2010). Besides the pure form of proteins and polysaccharides, those molecules also present in composite forms through covalent bonds such lectin-like protein (Higgins and Novak 1997), glycoprotein and proteoglycan-like compounds (Bourven et al. 2015). Different studies on EPS in granular formation have been conducted for the granulation process of aerobic and anaerobic sludge in which the PN/PS ratio has been used as an indicator for process performances (Ma et al. 2012a). The roles of EPS have been believed to include but not limited to: (i) structural formation and maintenance of aggregates or biofilm; (ii) increase of the substrate diffusivity; (iii) influence aggregate morphology by EPS hydrophobicity and (iv) the composition of EPS might influence biofilm or granule formation (Flemming and Wingender 2010; Vlaeminck et al. 2010). It is still arguable whether the production of EPS is the reflection of high or low microbial activity. Because the excretion of intracellular material could be a survival strategy under unfavorable condition, the enhanced extracellular enzymatic activity and/or mechanism to facilitate cell aggregation (Liu et al. 2004).

Physical and chemical or combinations of both methods have been developed to extract EPS from pure or mixed microbial aggregates. Physical methods include centrifugation, sonication, cation exchange resin (CER) and heating. Chemicals used for EPS extraction include alkaline, EDTA, ethanol etc. The selection of methods depends on sludge source as well as the parameters to be analyzed. Sheng et al. (2010) summarized the main extraction methods and their mechanisms in a review paper. It was concluded that CER has become the most widely applied method due to its high efficiency, low cell lysis and convenience in solid/liquid separation for EPS extraction from floccular and granular sludge (Sheng et al. 2010). Bourven et al. (2013) studied the fingerprints of EPS extracted by different physical and chemical methods on different types of sludge after separation by SEC columns. The conclusion is that physical methods only affect the relative absorbance of the fingerprints while chemical methods affects the fingerprints of EPS molecules as reflected by the abnormal peaks appeared on the chromatograms. Thus the physical method CER was chosen in this study.

A number of EPS characterization methods have been proposed to study the composition and their role in biological systems. Quantitative methods provide information about global concentration of proteins, humic-like substances, polysaccharides, uronic acids, nucleic acids, etc. To obtain more detailed characteristics of EPS molecules, various qualitative methods

have been developed including gas chromatography, Fourier transform infrared spectroscopy (FT-IR), size exclusion chromatography (SEC), 3-dimensionalexcitation-emission matrix fluorescence spectroscopy (3D-EEM), UV detection, confocal laser scanning microscopy (CLSM), etc. (Sheng et al. 2010). Among these, 3D-EEM appeared to be a simple and accurate method to identify organic compounds in water and wastewater (Kunacheva and Stuckey 2014). Chen et al. (2003) operationally defined excitation and emission wavelength boundaries into five regions based on literature survey, which provide the bases of identification of organic matter in different study (Bhatia et al. 2013; Bourven et al. 2012). As similar to those authors, the specific excitation/emission wavelength couples identified in 3D-EEM matrix are used for subsequent SEC analysis and UV detection in this study. SEC provides qualitative information about the distribution of EPS apparent molecular weight (MW) from sludge samples (Comte et al. 2007; Garnier et al. 2005; Gorner et al. 2003). UV detections at wavelengths of 210 nm, 254 nm and 280 nm, which assumed to correspond to different fractions of EPS molecules, are frequently used in literature (Bhatia et al. 2013; Ni et al. 2010; Simon et al. 2009). Hydrophobicity is also an important parameter in EPS characterization. Hydrophobic interaction plays a main role of sludge performance in settling, flocculation, and/or granulation. Determination of hydrophobicity is achieved by measurement of contact angle or by fractionation through XAD resin (Hou et al. 2015; Jorand et al. 1998; Martin Mousset et al. 1997).

Studies on EPS extracted from biological wastewater treatment system have been intensively conducted attempting to establish connection between reactor performance and EPS physic-chemical features. Those performances include (i) sludge settling and flocculation ability (Bala Subramanian et al. 2010; Wilen et al. 2003), (ii) biofilm and granular formation (Liu et al. 2004) and (iii) membrane fouling (Her et al. 2007), etc. Researches on EPS extracted from Anammox system have been mainly focused on the general composition change in response to salinity stress (Ma et al. 2012b; Windey et al. 2005). Chen et al. (2013b) studied influence of EPS composition and distribution on biomass activity using FT-IR spectra of sludge from a one-reactor partial nitritation/Anammox system without reaching any definitive conclusions on the relationship. Enrichment processes of Anammox biomass from different seeding sludge have been investigated by various authors (Chamchoi and Nitisoravut 2007; Kawagoshi et al. 2009; Wang et al. 2011). However, none of these studies covered the evolution of EPS composition and characteristics. Therefore, the aim of this work is to provide in-depth information regarding EPS characteristics in relation to different stage of

Anammox enrichment process and different performance level of enriched Anammox biomass.

4.2 Materials and Methods

4.2.1 Reactor operation

Seeding sludges were collected from the aeration tank and denitrification tank of a wastewater treatment plant (WWTP) treating municipal wastewater located in Nola, southern Italy. Aerobic sludge (ASR) and denitrification sludge (DSR) were seeded in two identical 5 L glass graduated cylinders operated as sequencing batch reactor (SBR) with working volume of 4 L. Synthetic wastewater was prepared according to de Graaf et al. (1996). Temperature was maintained at 34 ± 1 ℃ by thermostats. Feeding and discharging of influent and effluent was achieved by peristaltic pumps (Watson Malow, mod. 520Du and Velp Scientific, mod. SP311). Details of the operational conditions of the reactors could be found in Ding et al. (submitted). In brief, ASR was operated under condition with average dissolved oxygen (DO) level of 1 mg/L and a hydraulic retention time of 2 days. A total nitrogen removal reached a maximum of 60% after 240 days. DSR was operated with stricter oxygen control with DO level of 0.2-0.3 mg/L and a hydraulic retention time of 4 days. The total nitrogen removal of DSR reached a maximum of 80% after 150 days.

4.2.2 EPS extraction

Sludge was collected on day 0, 120 and 240 for ASR and day 0 and day 150 for DSR of the whole enrichment period for the investigation of EPS composition and patterns. The extraction was performed according to the slightly modified CER method provided by Frolund et al. (1996). In brief, the procedure is as follows: sludge was collected and centrifugated at 2000 g for 15 min to separate the solid and liquid phase. Supernatant was discarded and the sludge was resuspended to its original volume by EPS buffer with pH 7.0 ± 0.1 (2 mM Na_3PO_4, 4 mM KH_2PO_4 and 10 mM NaCl). Washed sludge and CER were mixed by a ratio of 70 gCER/gVSS and fully contacted by agitating with magnetic stirrer under 4 ℃ for 2h. EPS was extracted by centrifugation of the mixture at 16000 g for 15 min at 4 ℃. Extracted EPS was stored at -20 ℃ prior to further analysis.

4.2.3 Biochemical analysis

Global concentrations of protein and polysaccharide were measured by colorimetric method. Protein was measured by Lowry method (Frolund et al. 1996). Humic-like substance was not detected when modified Lowry method was applied (Frolund et al. 1995) probably due to the use of synthetic wastewater (Ni et al. 2009). Furthermore, fluorescence emission was never significantly observed in the specific area for humic-like substance in the excitation emission matrix proposed by Chen et al. (2003). Thus humic-like substance was not considered in this study. Polysaccharide was measured by phenol-H_2SO_4 method (Dubois et al. 1951). Total EPS was expressed by total organic carbon (TOC), which was measured by a TOC meter (Shimazu). All the measured results were normalized by TOC of EPS to facilitate comparison.

4.2.4 Excitation emission matrix (EEM) fluorescence spectroscopy

EEM spectra were determined through a spectrofluorometer (Shimadzu RF-5301 PC) to predefine the coupled excitation and emission wavelengths with the highest intensity. EPS samples were filtered by 0.45 μm filter and diluted by 50-100 times by 50 mM phosphate buffer (pH 7.0 ± 0.1) to achieve a proper concentration for the spectrofluorometer. Temperature was strictly controlled at 20 ± 1℃ in a thermostatic room. Emission was recorded from 225 to 550 nm at each excitation increment of 10 nm which range from 220 to 400 nm. The 3D EEM graphs were obtained by the software Panorama Fluorescence 3.1 (LabCognition, Japan) where X-axis represents the emission spectra from 220 to 550 nm, Y-axis as the excitation wavelength from 220 to 400 nm and color as the third dimension for emission intensity.

4.2.5 Size exclusion chromatography (SEC) analysis with diode array detector and fluorescence detector

Separation upon mass characteristics was performed by a set of instruments including (i) Merck Hitachi LA Chrom chromatograph equipped with a diode array UV detector (L7455), (ii) a fluorescence detector (L7485), (iii) an auto sampler (L7200), (iv) a quaternary pump (L7100) and (v) an interphase (D7000). Separation was performed by passing 100μL injection

volume of filtered sample (0.22μm, Sartorius) through two size exclusion columns connected in series. The columns refer to a high molecular weight column (Agilent Bio SEC-3 column 300Å, MW range: 5-1250 kDa) and a low molecular weight column (Agilent Bio SEC-3 column 100Å, MW range: 0.1-100 kDa). Phosphate buffer (150 mM NaCl, 25 mM Na_2HPO_4, 25 mM NaH_2PO_4, pH 7.0 ± 0.1) with a constant flow of 0.7 mL/min was used as mobile phase.

A specific wavelength of 210 nm was selected for UV detection in this study. The same couple of excitation/emission wavelengths were selected for SEC analysis for all the EPS samples for fluorescence detection according the 3D-EEM spectra where the area of the highest intensity was recorded. The following emission/excitation wavelength couples are elected: 221/350, 222/300 and 280/330 nm. All the absorbance and fluorescence signals, except that in the 3D-EEM, were normalized by the exact TOC of the injected EPS samples to facilitate comparison.

Calibration was performed according to the method described by (Bourven et al. 2012; Simon et al. 2009).Protein standards with different molecular weight were injected in HPLC-SEC, including Tyroglobulin (Fluka) – 660 kDa, Ferritine (Sigma) – 440 kDa, thyrotropin releasing hormone (Fluka) – 362 kDa, tyrosine (Fluka) – 181 kDa, Immunoglobulin G (Sigma) – 155 kDa, ovalbumine (Sigma) – 45 kDa, and ribonuclease (Sigma) A – 13.7 kDa. The logarithm of the molecular weight in Dalton shows a linear relationship with the elution volume. The plotted curve is regressed to the following equation (eq. 3):

$$\text{Log MW} = -0{,}2912\text{Ve} + 6{,}3841 \quad (R^2 = 0{,}9734) \qquad \text{(eq. 3)}$$

where MW is the molecular weight (kDa) and Ve is the elution volume (mL). The total permeation volume of the two series connected columns is 22 mL which was determined with NaN_3.

4.2.6 Hydrophobicity test

The hydrophobicity of the EPS sample was determined by the percentage of hydrophobic fraction in the total organic amount expressed by TOC. The hydrophobic fraction was

separated by passing the sample through Amberlite XAD 8 resin (Sigma) filled in an aluminum column. The procedure applied in this study was modified and simplified according to Jorand et al. (1998). Since our EPS samples had low concentration, a pre-precipitation step was not conducted. In brief, XAD 8 was submerged in distilled water adjusted at pH 5.0 ± 0.1 before using. EPS samples were adjusted to pH 5 ± 0.1 and filtered by 0.45 μm nitrate filter (Sartorius) prior to any analysis. The column filled with XAD 8 was connected to a peristaltic pump to allow a constant sample flow rate of 50 mL/h. The hydrophobic fraction was absorbed by the resin while the hydrophilic fraction passed through the column. By comparing the TOC before and after the resin, the percentage of the hydrophobic part could be calculated as follow (eq.4):

% hydrophobicity at pH 5.0 =100 x ($TOC_{before} - TOC_{after}$)/$TOC_{before}$ (eq. 4)

4.3 Results and discussion

4.3.1 Quantitative analysis of EPS components for ASR and DSR

The evolution of EPS components and EPS extraction yield as function of sludge dry weight is presented in Fig. 4.1. Due to the slow growth and the possibility of growth independent metabolism (Monballiu et al. 2013), volatile suspended solids (VSS) of the total biomass showed a decrease tendency of all the reactors Ding et al. (submitted). Despite that, EPS extraction yield of ASR sludge slightly increased while that of DSR sludge showed a significant increase of 37% (Fig. 4.1a). This might indicate that the EPS extraction yield is more correlated to microbial activity and reactor performance than sludge VSS. It was reported that EPS from Anammox bacteria showed a high protein content (Hou et al. 2015). However in the current study (Fig. 4.1b), the highest protein content was observed in the beginning of the enrichment in ASR followed by a sharp decrease in the middle and a further increase by the end. For polysaccharide, a slight decrease was observed from day 0 to day 120 in ASR and a sharp increase occurred by the end. The same trend of both components was found in DSR. PN/PS ratio decreased for both reactors, which indicate the role of exopolysaccharide in Anammox reactors. As a reactor with better performance, the amount of polysaccharide exceeded protein by the end of the enrichment. This is contradictory with the study of Chen et al. (2013b); Yin et al. (2015) who claimed that protein was dominant EPS

component in Anammox biomass. Also, the PN/PS values of ASR and DSR are in the lower range reported in literature, which is ranging from 1.22 to 3.1 (Chen et al. 2013a; Hou et al. 2015; Yin et al. 2015). The difference in trend and composition of EPS components between ASR and DSR may be due to the operation conditions as well as the different seeding sludge used. On day 0, DSR showed almost two times higher EPS extraction yield than ASR and a slightly higher PN/PS ratio is reported. Furthermore, denitrification sludge was proved to be more suitable for Anammox enrichment than aerobic sludge (Mulder et al. 1995).

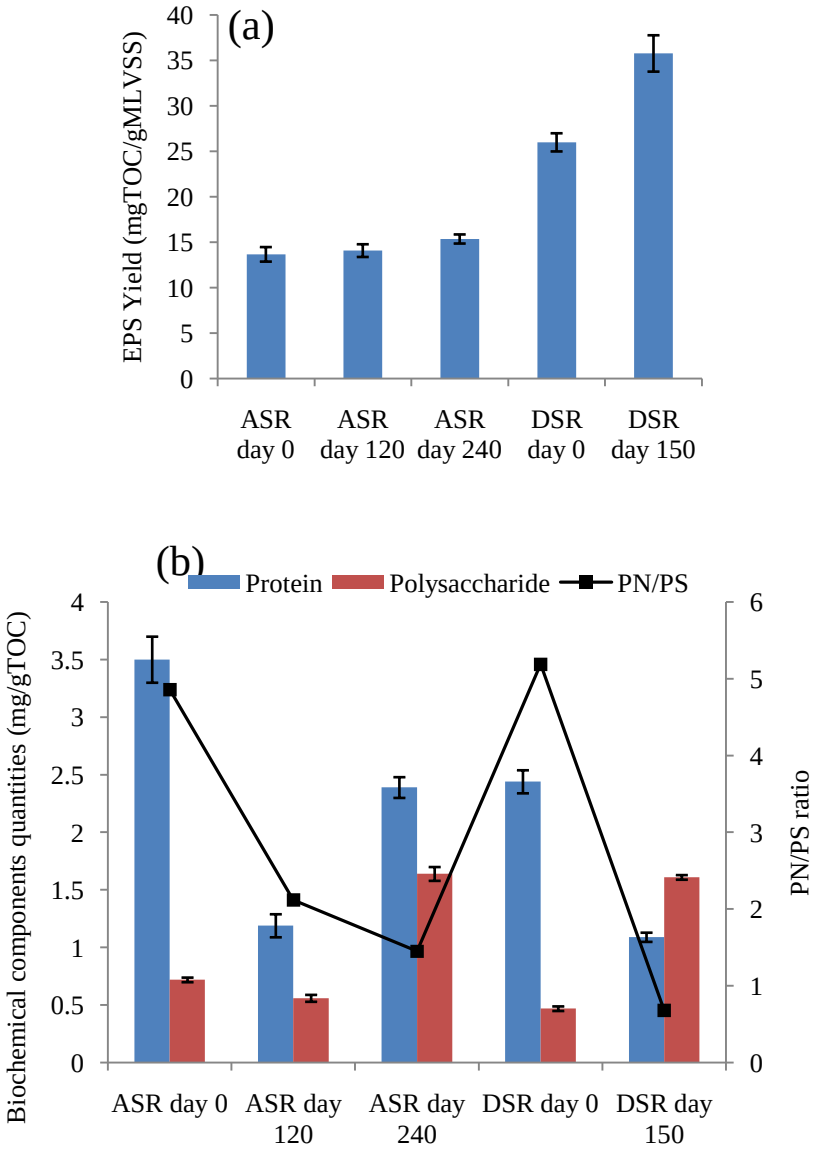


Fig.4.1. a) comparison of EPS extraction yield; b) Comparison of protein, polysaccharide and PN/PS ratio of EPS

4.3.2 Interpretation of 3D-EEM fluorescence spectra

Fluorescence of EPS molecules investigated by multiple excitation of different frequency was recorded in 3D-EEM spectra for all the EPS samples (Fig.4.2). Since quantification analysis is not the purpose of this study, the intensity presented in the matrix was not normalized by TOC for simplicity. EPS extracted from ASR showed similar peak location at about Ex/Em 220/300, 221/350 and 280/330 nm (Fig.4.2 a, b and c). EPS extracted from DSR had peak location slightly different from that of ASR (Fig. 4.2d), probably due to the different origin of the sludge sampled for extraction. Similar peaks were identified by Bourven et al. (2012)and Bhatia et al. (2013). However, Fig. 4.2a, b and c show two distinct peaks in zone I and II as defined by Chen et al. (2003) which corresponded to tyrosine- and tryptophan-like proteins. This is not the case for the seeded activated sludge (Fig. 4.2a) nor for other activated sludge (Wei et al. 2012), aerobic granular sludge (Adav and Lee 2011) or anaerobic granular sludge (Bourven et al. 2012). The separation of the peaks in zone I and II depends on various factors. For example, the location of tyrosine and tryptophan in the amino acids sequence as well as the protein structure and dynamics could affect the energy transferring from phenylalanine and tyrosine to tryptophan after excitation (Lakowicz 2013). Thus if large amounts of free amino acids and polypeptides are present in the sample, the condition may favor the separation of peaks for tyrosine and tryptophan. Therefore, it could be assumed that the separation of the peak was due to the specific structure of EPS molecules extracted from Anammox sludge. The peak locations of each EEM and their normalized intensity are presented in Table 4.1. It could be observed for both reactors that only very slight deviation of the peak from day 0 to the end of the enrichment period existed. Although the same protocol for extraction and sample preparation was applied, such deviation could be due to the slight environmental or instrumental disturbance.

Table 4.1 Peak location and intensity of EEM (normalized to TOC).

Sample	Zone I: tyrosine-like			Zone II: tryptophan-like			Zone IV: SMP-like		
	Ex (nm)	**Em (nm)**	**Intensity (AU/g of C)**	**Ex (nm)**	**Em (nm)**	**Intensity (AU/g of C)**	**Ex (nm)**	**Em (nm)**	**Intensity (AU/g of C)**
ASRday	220	300	1224.9	no peak			280	331	471.8

0									
ASR day 120	220	295	1073.4	220	338	1008.0	280	339	472.4
ASR day 240	220	297	1165.3	220	328	1151.1	280	338	554.2
DSR day 0 d	220	301	304.7	230	321	248.3	270	352	129.1
DSR day 150	230	297	435.7	230	331	454.9	270	328	202.5
							270	353	207.0

The interpretation of EEM requires statistical tools to treat complex multi-dimensional data. Two methods namely Fluorescence Regional Integration (FRI) and Parallel Factor Analysis (PARAFAC) have been adopted by different authors for the analysis of dissolved organics from natural and wastewater (Guo et al. 2015; Murphy et al. 2013). FRI was firstly examined by Chen et al. (2003) to analyze dissolved organic matter from river, drinking water and wastewater. FRI is based on an empirical delineation of EEM into five zones which correspond to different types of fluorophores. It also assumed that each fluorophore possesses only one peak in the scanned EEM ex/em wavelength. Under these two assumptions, the volume under the surface at each zone was integrated and calculated as percentage of the total zone. This percentage gave information about the weighing of each component in the organic compound (Chen et al. 2003). FRI, especially the ex/em boundaries, has been widely applied in the characterization of soluble microbial byproduct (SMP) and EPS from WWTP origin (Bhatia et al. 2013; Ni et al. 2009). However, PARAFAC showed that dual- or multi-excitation peak of the same fluorophore is possible (Fellman et al. 2009) and the phenomenon was confirmed by HPLC/HPSEC-FLD by Li et al. (2013). Furthermore, the emission wavelength of some organics such as protein is sensitive to pH, temperature, denaturing of the protein structure as well as quenching by other molecules (Lakowicz 2013). The latter two factors are generally unpredictable for dissolved organic carbon (DOC) samples. Thus further characterization on the obtained peak intensity data from EEM should be treated carefully. In the presented study, it is possible that multi-excitation peak occurred in region I, II and IV because very similar SEC chromatograms were observed (Fig. 4.3 and 4.4).

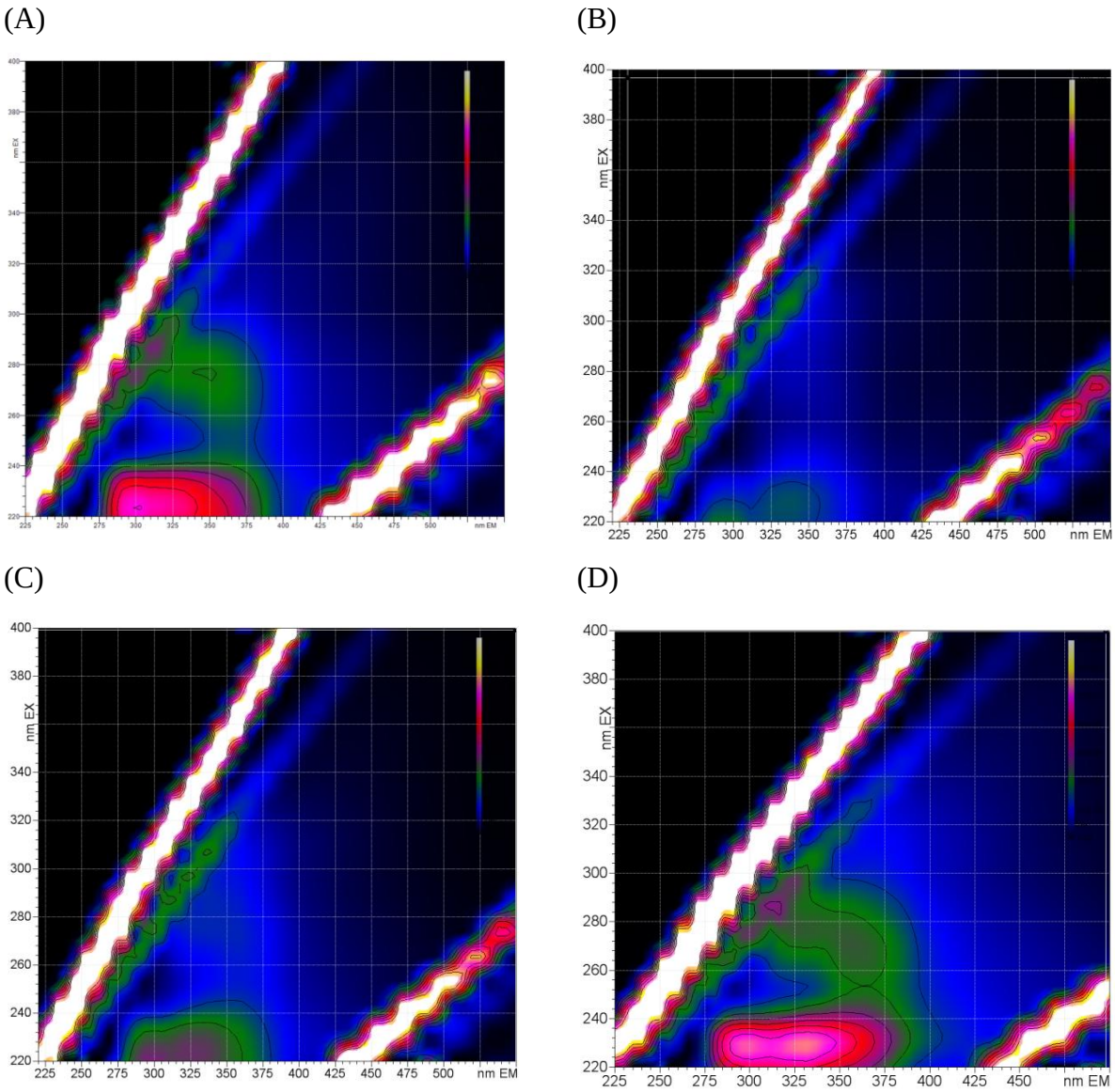


Figure 4.2 Fluorescence EEM of EPS diluted in phosphate buffer at pH 7.0, 20℃. (A) Sampled on day 0 from ASR; (B) Sampled on day 120 from ASR; (C) Sampled on day 240 from ASR; (D) Sampled on day 150 from DSR.

4.3.3 Evolution of SEC fingerprints of ASR recorded by UV and fluorescence detector

The UV chromatogram after SEC elution was based on the principle of UV absorption in which electron transition of chromophores occurred from its highest occupied molecular orbital (HOMO) to lowest unoccupied molecular orbital (LUMO). Upon the absorption of a photon, ground state electron of σ or π orbital from covalent bounds or electron from nonbonding orbital (n) are excited to antibonding σ* or π* orbital (Pretsch et al. 2013). At the wavelength of 210 nm which was chosen in this study, the following electron transitions

mostly frequently occur as calculated from the respective energy required: i) $\pi \rightarrow \pi^*$, which exists predominantly in conjugated double bonds such as unsaturated aliphatics and aromatics; ii) $n \rightarrow \sigma^*$, which is typical for compounds containing nitrogen, sulfur, phosphorous or halogens such as amine, hydroxyl, thiol and chloride; iii) $n \rightarrow \pi^*$, which happens in carboxylic acid derivatives and phenol like compounds (Pretsch et al. 2013). Thus in the case of EPS sample, compounds which possess high absorption at 210 nm represent most aliphatic and aromatic polysaccharides due to the large amount of antibonding σ^* and conjugated π^* orbital and nonbonding n orbital, whereas proteins are less represented as most proteins have highest absorption at 280 nm (Lakowicz 2013). Fluorescence is a more sensitive approach for protein characterization. Aromatic amino acids, namely phenylalanine, tyrosine and tryptophan, are the fluorophores among the 20 amino acids. The wavelength at which emission maximum is recorded is 282 nm for phenylalanine, 303 nm for tyrosine and 350 nm for tryptophan. Fluorescence for phenylalanine could hardly be detected because of its low quantum yield (Lakowicz 2013) thus is not considered in this study. From Fig. 4.3, it could be observed that the maximum fluorescence was detected at ex/em 220/300 and 221/350 nm, which were consistent with the theoretical maximum emission wavelength but inconsistent with the maximum absorption wavelength of 280 nm. Thus for proteins, the "Mirror image rule" does not apply. Instead, Stoke's shift and emission independence of excitation energy may occur (Lakowicz 2013).

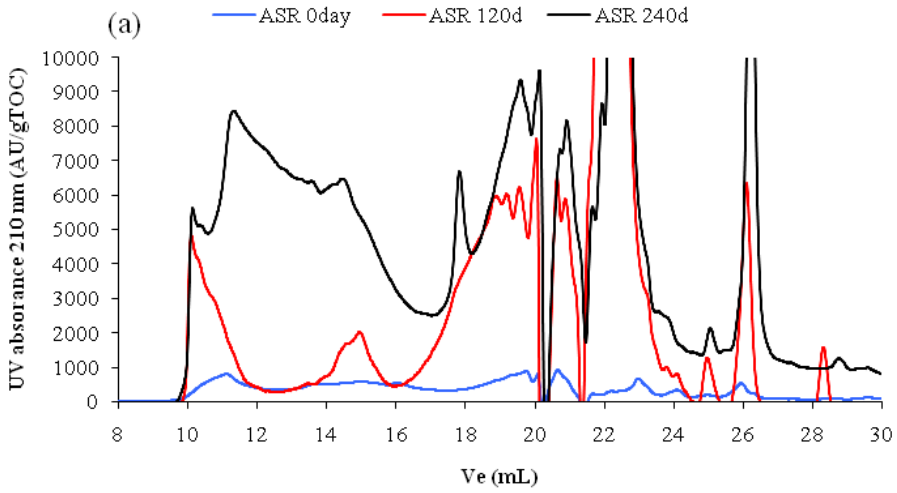
Fig. 4.3 shows the chromatograms of UV detection at 210 nm and fluorescence detection at ex/em 220/300 nm, 221/350 nm and 280/330 nm for EPS samples from day 0, 120 and 240 of ASR. On day 0, only two peaks were identified on the UV chromatogram (Fig. 4.3a) at elution volume of 11 mL and 16 mL. Those peak locations coincided with those from the fluorescence chromatograms (Fig. 4.3b, c, d). This implies that on day 0, EPS was dominated by protein whereas few polysaccharides were present, which is consistent with the quantitative analysis (Fig. 4.1b). This may be because on day 0 the sludge used for EPS extraction was actually activated sludge as the enrichment did not start. It was reported that EPS in activated sludge have much higher amount of proteins than polysaccharides (Ding et al. 2015). On day 120, Anammox enrichment was under "propagation phase", when aerobic ammonium and nitrite oxidation bacteria were inhibited and Anammox process was not fully established. Two major peaks were identified at 10 mL and 15 mL, while another three less separated peaks was found near the end of the total elution volume of 20 mL. The peak at 10 mL elution volume did not coincide with any fluorescence peaks of the same sample in Fig

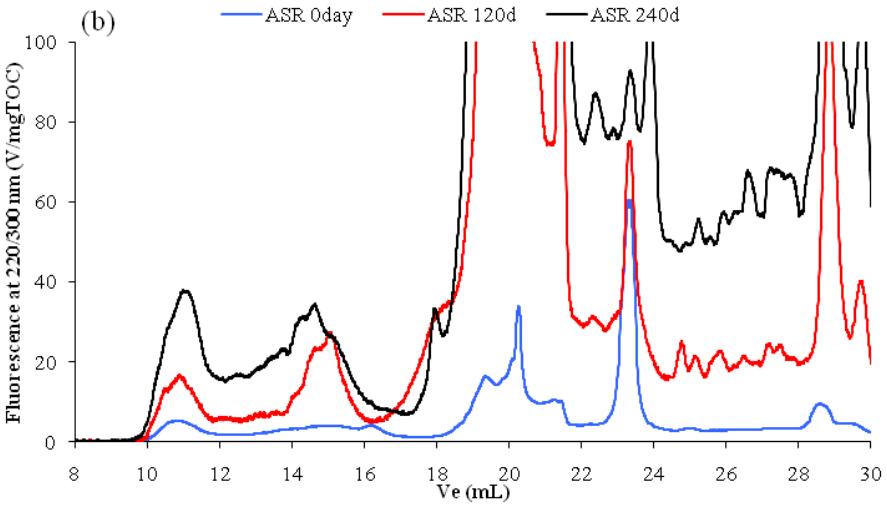
4.4b and c, which indicated the presence of other molecules structure, which was assumed to be polysaccharide according to the absorbance and fluorescence properties. Peak at 15 mL elution appeared at both UV chromatogram (Fig. 4.4a) and fluorescence chromatogram (Fig. 4.4b and c). This might indicate the development of protein-polysaccharide composites such as glycoprotein and proteoglycan. The high intensity of the peaks at small molecular weight range might be the result of digestion of high weight molecules by extracellular enzymes (Nielsen et al. 1996). On day 240, Anammox activity was established as confirmed by process parameters (Ding et al. submitted). Multiple peaks were identified on Fig. 4.4a at elution volume of 10, 11.5, 14.3, 18, 19.5 and 20 mL. This meant that polysaccharides with various molecular weights were produced due to the establishment of Anammox process. The increase of the area under the curves corresponded to the decrease of PN/PS ratio of ASR (Table 4.1). These results were inconsistent with some studies in which a high PN/PS ratio was found (Chen et al. 2013b; Hou et al. 2015; Yin et al. 2015).

It could be observed from Fig. 4.3b, c and d that all the fluorescence chromatograms show similar feature with difference only in relative intensity as well as the ratio of the under curve areas of the two major peaks. This might indicate the shift of molecular weight (MW) distribution and production of new protein molecules, either in composite or pure form, during the enrichment. Since extracellular proteins contain a considerable amount of enzyme, it could be assumed that the extracellular enzymatic activity might change with the increased activity of Anammox bacteria. Tryptophan had the highest emission intensity possibly due to the high amount of conjugated π bond and as well as the indole structure, which might potentially exhibit high absorbance and fluorescence. Furthermore, according to Lakowicz (2013), energy absorbed by phenylalanine and tyrosine are often transferred to tryptophan. Thus tryptophan fluorescence could be considered most representative for protein characterization. However, tryptophan is sensitive to solvent effects and prone to quenching by protein side chains, which is a limitation of the method. The under curve areas showed increase trend in all chromatograms. However this is inconsistent with the protein concentration presented in Table 4.1. Since the protein concentration is measured based on the quantification of peptide bonds in the applied Lowry Method (Frolund et al. 1996), the decrease in total protein concentration refers to decrease of the amount of peptide bonds. On the other hand, the increase in the under curve area of tryptophan fluorescence chromatogram (Fig. 4.3c) indicated an increase of amino acids. Thus the discrepancy might indicate the structural shift of EPS molecules, for example from protein dominated by the increase of

glycoprotein proportion at the same molecular weight. According to the delineation of the EEM by Chen et al. (2003), fluorescence present in zone IV represents the "soluble microbial byproduct-like" (SMP) compounds. The EEM of the samples all exhibited fluorescence in zone IV and the ex/em wavelength couple of 280/330 nm was selected for further characterization by SEC. The chromatogram obtained at ex/em 280/330 nm (Fig. 4.3d) was almost identical to the tryptophan chromatogram (Fig. 4.3c) except lower in intensity. Furthermore, there was no clear explanation of SMP in the study of Chen et al. (2003). Thus it seems reasonable to assume that the SMP and extracellular protein overlapped and multi-excitation peaks occurred because the fluorescence emission of protein is independent of its excitation wavelength.

As mentioned above, fluorescence of tryptophan is representative for protein characterization. The discussion on the trend of chromatogram during evolution mainly focuses on the tryptophan chromatogram (Fig. 4.3c). Two major peaks were identified for all samples at elution volume of 10.7 mL and 15 mL. On day 0, low intensity was detected even at the end and after the total elution volume probably due to the low microbial activity. On day 120, an increase in intensity occurred of both HMW and LMW range with more significant increase in LMW range. This might be due to the growth and aggregation of Anammox bacteria during the propagation phase. Day 240 exhibited further increase in HWM range while slightly decrease in the LMW range, probably as a result of established high Anammox activity and cell aggregation, which was believed to be significantly contributed by extracellular protein (Hou et al. 2015). Fluorescence chromatograms at ex/em 220/300 nm and 280/330 nm showed very similar trends as ex/em 221/350. If SMP is concerned, the increase of intensity at 280/330 nm may indicate the increase of microbial activity which might be related to shift of microbial diversity and Anammox activity. The statement is based upon the definition of SMP by Laspidou and Rittmann (2002) in which SMP is composed of substrate-utilization-associated products (UAP) and biomass-associated products (BAP). UAP may refer to the Anammox activity which converted ammonium and nitrite to nitrogen gas while BAP may result from the decay of AOB, NOB and denitrifiers due to the unfavorable operation condition.


(a)
ASR 0day
ASR 120d
ASR 240d
UV absorance 210 nm (AU/gTOC)
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
8
10
12
14
16
18
20
22
24
26
28
30
Ve (mL)


(b)
ASR 0day
ASR 120d
ASR 240d
Fluorescence at 220/300 nm (V/mgTOC)
100
80
60
40
20
0
8
10
12
14
16
18
20
22
24
26
28
30
Ve (mL)

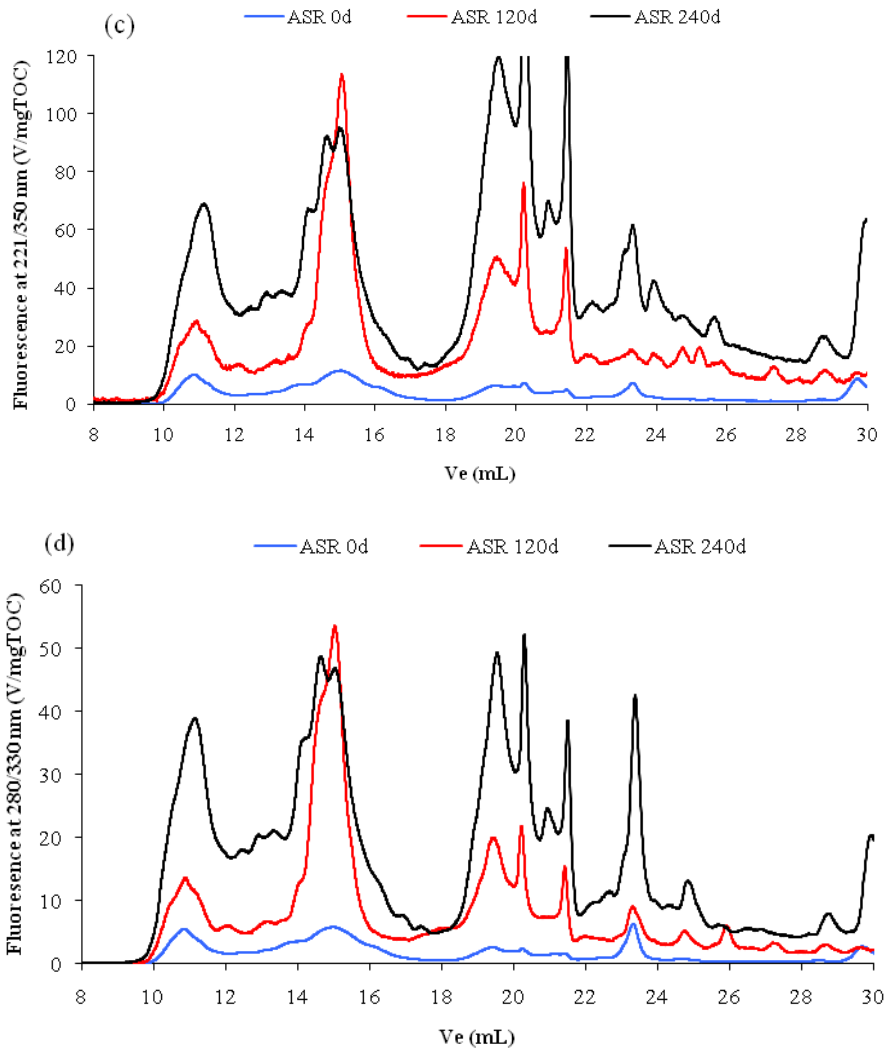
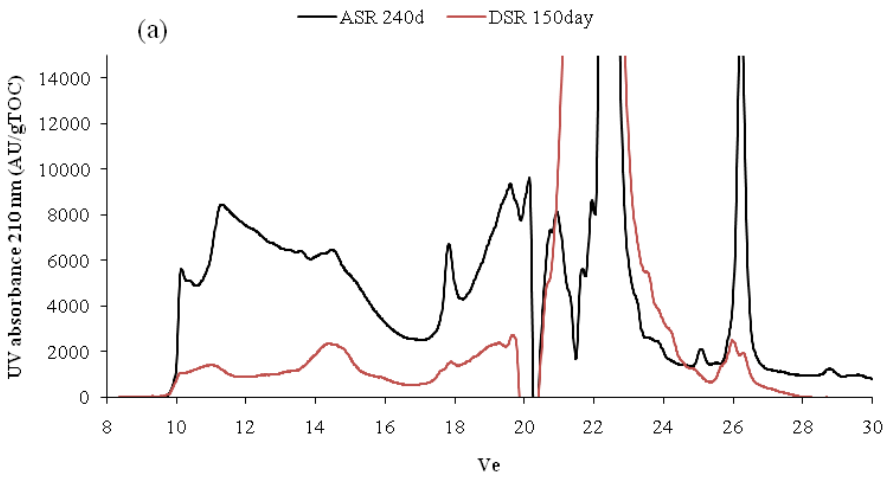


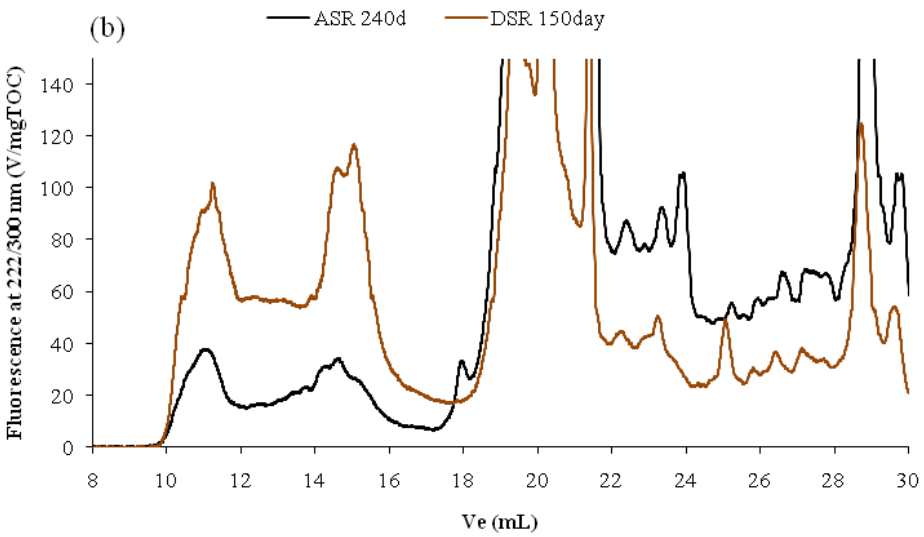
Fig. 4.3 Evolution of UV and fluorescence chromatograms after SEC for ASR. (a) UV absorbance at 210 nm; (b) Fluorescence detection at ex/em 222/300 nm; (c) Fluorescence detection at ex/em 221/350 nm; (d) Fluorescence detection at ex/em 280/330 nm.

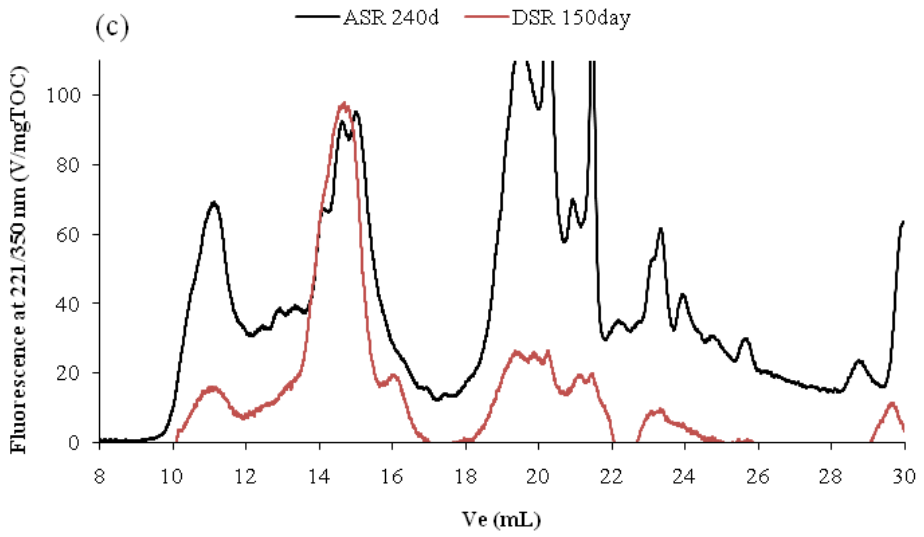
4.3.4 Comparison of SEC chromatograms between EPS extracted from ASR and DSR

UV and fluorescence chromatograms of ASR and DSR at the end of the enrichment period were presented in Fig. 4.4 for comparison. The major differences between ASR and DSR are represented by the seeding sludge used for cultivation and the strictness of process control especially in the control of DO, which resulted in better total nitrogen removal for DSR. A higher UV absorbance of ASR was observed in Fig. 4.4a while the protein and polysaccharide content was similar according to quantitative analysis (Table 4.1). This might imply that some organics may be present in EPS extracted from DSR that show no absorption at wavelength of

210 nm, for example alkene and non-conjugated dienes have maximum absorption below 200 nm (Clark et al. 1993). The different peak locations also indicate the different composition of EPS between two samples. In tryptophan fluorescence chromatogram (Fig. 4.4c), EPS extracted from ASR showed higher intensity at HMW range and similar intensity with DSR at LMW range. Fluorescence intensity of DSR even showed low intensity at the end and after the total elution volume, which implied the small content of low weight molecules such as amino acids. Chromatograms obtained at ex/em 280/330 nm were nearly identical with that at ex/em 221/350 nm, which meant multi-excitation peak also occurred for EPS sample from DSR. As different from ASR, fluorescence chromatogram obtained at ex/em 222/300 nm showed high intensity for EPS from DSR. This might imply that tyrosine presented in high amount in extracellular protein of DSR. All the fluorescence chromatograms showed same peak location but differed in intensity as well as ratio of under curve area of each peak, indicating the difference structure of macromolecules between the two EPS samples. Above all, it is not yet possible to correlate the reactor performance and type of seeding sludge with EPS pattern in this study. As stated by Ding et al (2015), it is difficult to correlate process in different reactors with quantitative or qualitative EPS characterization whereas change of EPS size and composition features is indicative for comparison of process within one reactor.




(b)
ASR 240d
DSR 150day
Fluorescence at 222/300 nm (V/mgTOC)
0
20
40
60
80
100
120
140
8
10
12
14
16
18
20
22
24
26
28
30
Ve (mL)


(c)
ASR 240d
DSR 150day
Fluorescence at 221/350 nm (V/mgTOC)
0
20
40
60
80
100
8
10
12
14
16
18
20
22
24
26
28
30
Ve (mL)

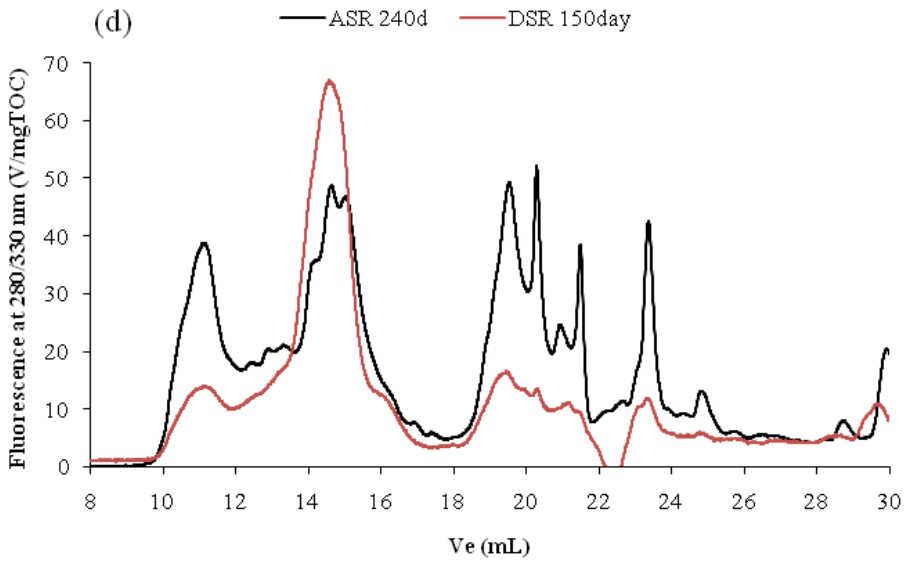

(d)
ASR 240d
DSR 150day
Fluorescence at 280/330 nm (V/mgTOC)
0
10
20
30
40
50
60
70
8
10
12
14
16
18
20
22
24
26
28
30
Ve (mL)

Fig. 4.4 Comparison of chromatograms of ASR and DSR. (a) UV absorbance at 210 nm; (b) Fluorescence detection at ex/em 222/300 nm; (c) Fluorescence detection at ex/em 221/350 nm; (d) Fluorescence detection at ex/em 280/330 nm.

4.3.5 Evolution and comparison of hydrophobicity

An estimation of the hydrophobicity of the EPS samples from Anammox reactors were obtained through the use of Amberlite XAD 8 resin. The pH of the samples was adjusted to 5 instead of 2, as suggested by Jorand et al. (1998) as a compromise to maintain the acidity while avoid the significant precipitation of the proteins. However at pH 5, the sorption of the organic compounds is no longer a certain function of their solubility (Aiken et al. 1992; Jorand et al. 1998). Thus the results presented here are just an estimation of the evolution of the EPS hydrophobicity (Fig. 4.5). It could be observed that the EPS hydrophobicity increased with the Anammox enrichment process. According to Innerebner et al. (2007), Anammox bacteria tend to grow in aggregates such as flocs, granules or attached to the reactor wall to form biofilm. As proposed by Hou et al. (2015), hydrophobicity is the main driving force determining the aggregation of Anammox microorganisms. Thus it is reasonable to assume that the increase of hydrophobicity is the result of such aggregation property of the enriched Anammox biomass. However, the EPS hydrophobicity did not positively correlated to the reactor performance between ASR and DSR since the hydrophobicity of DSR at the end of the enrichment process resulted lower than ASR. Thus further calibration of the protocol as well as characterization of more samples from other reactors shall be conducted to draw a more confirmative and representative conclusion.

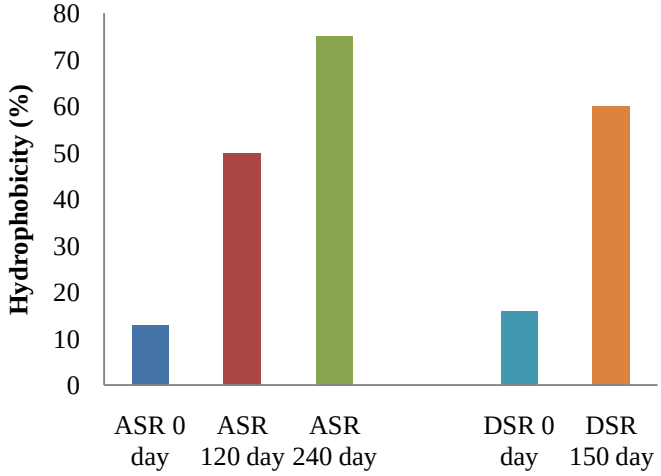


Fig. 4.5 Hydrophobicity of EPS samples

4.4 Conclusions

The paper attempts to provide in-depth information regarding the EPS extracted from Anammox biomass collected at different enrichment stage and different process performance. A series of quantitative and qualitative analysis was conducted to investigate the differences in EPS characteristics. The following conclusions could be drawn:

- Quantitative measurements gave global information on the amount of protein and polysaccharide contained in EPS extracted from Anammox biomass collected at different stage of the enrichement. However, a clear correlation between the concentration of each component and process performance was not found. Furthermore, colorimetric methods could not give information about the protein-polysaccharide composite such as glycoprotein and proteoglycan which are commonly found in EPS samples (Bourven et al. 2015). Thus the expressed value of protein and polysaccharide might be overlapped to some extent.
- The 3D-EEMs showed that EPS extracted from sludge under or after Anammox enrichment display two distinct peaks at zone I and II according to the delineation by Chen et al. (2003). Whereas the EEM obtained for activated sludge anaerobic granular sludge mostly possess only one peak (Adav and Lee 2011; Bhatia et al. 2013; Sheng and Yu 2006). This might due to the specific composition or structure of EPS extracted from Anammox enriched sludge. Multi-excitation peak might occur as evidenced by the identical fluorescence SEC chromatogramat ex/em of 221/350 and 280/330 nm.
- A clear difference in the UV chromatograms during the enrichment of Anammox, in terms of peak location, intensity and number of peaks was noticed. It showed increases in both the intensity and number of peaks, indicating that more diverse molecular structure occurred as a result of Anammox enrichment. Fluorescence SEC chromatograms of all selected ex/em wavelength couples showed similar shape with differences in intensity. However correlation between the performance of Anammox activity (i.e. ASR and DSR) and the chromatograms could not be established.
- An increase of hydrophobicity was observed for both ASR and DSR presumably due to the aggregation of Anammox bacteria. However further calibration of the method as

well as other information such as the morphology of Anammox sludge need to be revealed to reach a confirmative conclusion.

Both quantitative and qualitative characterization could give valuable information about EPS extracted from Anammox sludge. Besides those presented in this paper, further studies are expected especially on the fractionation, purification and molecular characterization of the peaks in the chromatograms.

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Chapter 5

An Innovative Approach to Remove Nitrogen from Wastewater Using a Biological ANaerobic AMMonium OXidation (ANAMMOX) Process

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Chapter 5 An Innovative Approach to Remove Nitrogen from Wastewater Using a Biological ANaerobic AMMonium OXidation (ANAMMOX) Process

Summary

Nitrogen (N) is essential for life because it is one of the main constituents of organic molecules such as amino acids and proteins.However, at the same time it is a threat to both the environment quality and human health. To avoid the negative effects of N, recent worldwide regulations have been promulgated to limit the use of nitrogen based fertilizers and the amount of nitrogen discharged into water bodies from wastewater treatment plant (WWTP). An aerobic oxidation of reduced N either followed or preceded by an anoxic reduction of oxidized N are the most common systems used in WWTP to convert the reactive N (ammonium, nitrite and nitrate) to the harmless nitrogen gas (N_2). An alternative to the traditional systems is represented by the innovative biological process named Anammox that anaerobically converts ammonium into N_2. Compared to the traditional processes, this new process requires lower amount of oxygen, has no need of organic carbon supply and is highly efficient with any concentration of ammonium. However, a wide use of this process at full scale is hampered by the extremely slow growth rate of bacteria operating Anammox process and by the difficulty in the control of critical co-existence of different bacterial strains that compete for the same substrate and thrive in opposite environmental conditions. This work highlights the strategies to improve the enrichment of Anammox operating bacteria from two different biological sludge (activated and anaerobic) and to optimize the efficiency of two Anammox biological sequential batch reactors (SBR) by changing the operational conditions. The main results have shown that in an activated sludge the Anammox biomass grows faster than in anaerobic sludge and the performance of Anammox one-stage process can be regulated by controlling the stirring system as well as the oxygen and inorganic carbon (IC) concentrations in the system even if no one of the previous operating parameters has resulted to be decisive.

5.1 Introduction

The intensive use of nitrogen(N)-based fertilizers for increasing the productivity of cultivated fields (Pindozzi et al., 2013) as well as the discharge of wastewaters rich in N-based compounds into surface waters have distorted the equilibrium of the natural N cycle (Infascelli et al., 2010). To prevent further excess introduction of N-based compounds into the environment, it is therefore mandatory to intervene in limiting the main sources of N (i.e. agricultural fertilizer use and wastewaters discharge). Regarding the wastewaters, most of the currently operating treatment plants have been recently upgraded by adding a tertiary biological treatment with the aim of removing residual N-based compounds. The conventional processes used in wastewater treatment plants (WWTP) for this purpose achieve the complete oxidation of ammonium to nitrate in the presence of oxygen in the first step (nitrification) and the conversion of nitrate into the inert N_2 under anoxic conditions in presence of a sufficient amount of biodegradable organic material (already present in the wastewater or added from an external source if required) in the second step (denitrification). However, if ammonium (NH_4^+) is present in high concentrations in water, it becomes toxic and thus inhibits the activity of the microorganisms involved in its removal. Therefore, the conventional processes based on biological nitrification and denitrification of nitrogen compounds appear to be not suitable for high NH_4^+ load wastewaters. The operating limits shown by the conventional N-based compounds removal can be overcome by using a novel alternative biological process (Hu et al., 2013) which is furthermore able to outcompete them in terms of cost as well as removal efficiency. This process is named Anammox as performs an anaerobic ammonium oxidationby using special anaerobic ammonium oxidizing bacteria, capable of using nitrite as electron acceptor to directly convert NH_4^+ into N_2 gas in the absence of dissolved O_2 (Isaka et al., 2006) according to the following chemical reaction Eq. (1):

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- \rightarrow 1.02N_2 + 2.03H_2O + 0.066CH_2O_{1.5}N_{0.15} + 0.26NO_3^-$$

Eq. (1)

Bacteria performing the Anammox process are autotrophic and use CO_2 as carbon source. The O_2 demand for converting NH_4^+ to N_2 through partial nitritation/Anammox pathway is much lower than that through complete nitrification/denitrification pathway, since the O_2 is necessary only for a partial oxidation of the total NH_4^+ to nitrite (NO_2^-) (partial nitritation) conducted by autotrophic and aerobic ammonium oxidizing bacteria (AOB), whereas the remaining part of NH_4^+reacts with NO_2^- to form N_2 gas by Anammox bacteria (Fux et al., 2002). Compared with the conventional biological ammonium removal processes, the

Anammox process has no need of an external carbon source (Panepinto et al., 2013), furthermore shows a low yield of biomass and consequently a lower sludge production. These aspects, in addition to a lower O_2 demand, keep low the operation costs to perform an Anammox process. Moreover the emissions of CO_2 in the environment are lower than conventional treatments, thus this process results also in low environmental impact. This work presents preliminary results of experimental activities conducted on Anammox bacteria with the aim of accelerating the Anammox bacteria enrichment process from two different biomasses as well as improving the performance of a previously enriched Anammox biomass in a lab scale completely autotrophic nitrogen removal over nitrite (CANON) type reactor designed to remove NH_4^+ (Third et al., 2001). All the experiments were conducted in sequencing batch reactors (SBR) systems (Backburne et al., 2008). The most critical points of the Anammox process faced in this work were the slow growth rate of Anammox bacteria (Li et al., 2012) that requires long time of start-up and the antagonism (Mattei et al., 2015) between Anammox bacteria and AOB that compete for the same substrate (inorganic carbon and ammonium) and need opposite operating conditions (i.e. anoxic for Anammox bacteria, aerobic for AOB).

5.2 Materials and methods

The Anammox process was performed in 4 discontinuously fed SBRs, where the processes of biological oxidation and settling took place alternatively. Purified effluents were discontinuously discharged just before the feeding operation, whereas the excess sludge wasrecycled when necessary. All these processes and operations were performed according to a specific and cyclic time sequence.

5.2.1 Biomass collection and preparation

Two different biomasses were selected as seeding sludge to develop Anammox bacteria in two identical SBRs: the first reactor (AcS) was fed with 2 L activated sludge (1.2% in total solids-TS content, v/v) taken from the denitrification tank of a municipal WWTP, located in Nola (NA), southern Italy; the second reactor (AnS) was filled with 0.9 L sludge (2.7% in TS content, v/v) taken from an anaerobic digester treating buffalo manure and milk serum located in Albanella (SA), southern Italy. The remaining two SBRs (BRS1 and BRS2) were filled

with 0.1 L sludge (1.4% in TS, v/v) taken from a full-scalepartial nitritation-Anammox process at the municipal WWTP of Brunico (BZ), Italy.

5.2.2 Synthetic Wastewater preparation

The composition of the mineral medium used to feed all SBRs has been prepared following the recipe of van de Graaf et al. (1996). In detail, 0.5 g $KHCO_3$, 0.5 g $NaHCO_3$, 0.054 g KH_2PO_4, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.136 g $CaCl_2 \cdot 2H_2O$, 1 mL of trace element solution I and 1 mL of trace element solution II were added to 1L of demineralized water (Elga, PURELAB Option Q-series, Italy). Trace element solutions I was composed of 5 g/L EDTA and 5 g/L $FeSO_4 \cdot 7H_2O$. Trace element solution II was composed of 5 g/L EDTA, 0.43 g/L $ZnSO_4 \cdot 7H_2O$, 0.24 g/L $CoCl_2 \cdot 6H_2O$, 0.99 g/L $MnCl_2 \cdot 4H_2O$, 0.25 g/L $CuSO_4 \cdot 5H_2O$, 0.22 g/L $Na_2MoO_4 \cdot 2H_2O$, 0.19 g/L $NiCl_2 \cdot 6H_2O$, 0.21 g/L $Na_2SeO_4 \cdot 10H_2O$, and 0.014 g/L H_3BO_3. Synthetic wastewater was prepared by adding to the mineral medium required amounts of $(NH_4)_2SO_4$ and $NaNO_2$for Anammox biomass enrichment SBRs (i.e. AcS and AnS) and only $(NH_4)_2SO_4$for the other two SBRs (i.e. BRS1 and BRS2) where Anammox process was performed.

5.2.3 Anammox biomass enrichment reactors

Two identical 5L cylindrical glass SBRs with working volume of 4 L were used to perform the Anammox biomass enrichment. The operating anoxic conditions were achieved and maintained by hermetically closing the reactors and temporarily flushing them by inert Argon gas. A temperature of 34 ℃ was constantly maintained inside the reactors by an external thermostatic bath heated by thermostat heaters (Odyssea, heat pro 100, Canada). The hydraulic retention time (HRT) was set to 4 days. The cyclic exchanging volume was set to 1 L (25%). The working sequence lasted 24 hours and it was composed of 4 phases as follows: feeding time (80 minutes); reaction time (16 hours and 40 minutes); sedimentation time (4 hours); discharge time (20 minutes). A magnetic stirring system (Ika, C-MAG MS 10, Germany) was used to maintain the biomass suspended. Feeding and discharging operations were performed by peristaltic pumps (Watson Marlow, 520 Du, UK) to achieve desired flow according to the feeding and discharging scheme of different reactors described above.The on/off operations of all devices used in the experiment were regulated by an electronic timer

(GIB, HWD EG01, Germany). All the reactors were fed with synthetic wastewater (see sub-section 2.2) prepared every 2 days. The molar ratio of NO_2^--N/NH_4^+-N shifted between 0.76 and 1.5, in response of nitrite accumulation. Changes (i.e. amounts of nitrogen based compounds and their molar ratio) in the feeding solution were operated when the reactors experienced acceptable nitrogen removal efficiency (positive indication) or accumulation of undesirable compounds, i.e. nitrite and nitrate (negative indication). Before feeding, the synthetic wastewater was appropriately degassed by a vacuum pump (KNF Laboport, N 820 FT.18, Germany) to completely eliminate oxygen.

5.2.4 Partial nitritation-Anammox (CANON type) system reactors

The partial nitritation-Anammox (single stage) process was conducted in two SBRs with working volume of 2L, constituted by a 2L Schott bottles (Duran, Germany). The biomass was maintained in suspension by a mechanical stirrer (Guangzhou, mod. AM300S-H, China). The degradation of substrate (i.e. synthetic wastewater) was performed in alternating aerobic/anoxic conditions. A temperature of 34 ℃ was maintained constantly by an external thermostatic bath heated by thermostat heaters (Odyssea, heat pro 100, Canada). HRT was set equal to 4 days. The cyclic exchanging volume was set to 0.5 L (25%). The working sequence lasted 24 hours and it was composed of 4 phases as follows: feeding time (30 minutes); reaction time (22 hours); sedimentation time (1 hour); discharge time (30 minutes). Feeding (Watson Marlow, mod. Varmeca, UK) and discharging pumps (Watson Marlow, 302s, UK) were set with a hydraulic flow equal to 1.25 mL min^{-1}. The oxygen in the system was discontinuously (30 minutes for cycle) supplied by micro pore air diffusers (Ecoplus, aquarium cylinder, UK) with an air flow of 0.5 L min^{-1}. The on/off operations of all devices used in the experiment were regulated by an electronic timer (GIB, HWD EG01, Germany). The reactors used to perform partial nitritation-Anammox system were fed with synthetic wastewater (see subsection 2.2) where ammonium was added in concentration that was increased during the investigation time according to the efficiency level in nitrogen removal achieved by the system. To limit nitrate accumulation the composition of synthetic wastewater was changed during the experiment as well as the operating conditions.

5.2.5 Analytical methods

The ammonium concentration (mg NH_4^+-N/L) was measured by Nessler method using a spectrophotomer (WTW, PhotoLab 6600 UV-VIS series, Germany) as well as by distillation equipment (Velp Scientifica, UDK 132 Semiautomatic Distillation Unit, Italy) when concentration is higher than 3 mgNH_4^+-N/L (APHA standard methods, 2005). Nitrite and nitrate concentration (mg NO_2^--N/L, mg NO_3^--N /L, respectively) were measured by ionic chromatography device (Metrohm, 761 Compact IC, Switzerland) and spectrophotometric equipment (WTW, PhotoLab 6600 UV-VIS series, Germany). The pH was measured by a portable pH meter (WTW, inolab, Germany). Total and carbonate alkalinity were determined by titration according to Anderson and Yang (1992). Titrations were performed by using an automated titrator (Radiometer Copenhagen, TT80, France).

5.3 Results and discussions

5.3.1 Anammox biomass enrichment

Both reactorswere initially fed with a total nitrogen loading rate (NLR) of 0.01 kgN m^3 d^{-1} by pumping into the reactors a solution containing 26 mgNO_2^--N /L of $NaNO_2$and 20 mgNH_4^+-N/L of $(NH_4)_2SO_4$ according to a stoichiometric molar ratio of 1.32 (Eq. (1)). After day 8, theNO_2^--N/NH_4^+-N ratio was modified to 1.50 by increasing the NO_2^--N concentration to 30 mgNO_2^--N /L, with the aim of preventing a lack of NO_2^--N for Anammox bacteria (Figure 5.1). In this time interval the concentration of NH_4^+-N ranged in the effluent between 1.0 and 8.8 mg/L whereas the concentration of NO_2^-Nbetween 2.1 and 18.2 mg/L. From day 26 to day 44 the concentration of NH_4^+-N in the influent was increased up to23 mg/L, with the aim of establishing again the NO_2^--N/NH_4^+-N molar ratio to 1.32 and at day 45 the concentrations of NO_2^--N and NH_4^+-N were doubled (60 mg/L NO_2^--N and 46 mg/L NH_4^+-N). From day 40 the reactor AcS experienced an increase of nitrite concentration up to 65 mg/L NO_2^--N on day 78. This occurrence led to the decision to feed the reactor for the next 7 days with a solution containing only NH_4^+-N at concentration of 46 mg/L since nitrites in high concentrations are considered toxic for Anammox (Lotti et al., 2012). Nitrite accumulation was probably due to presence in the sludge of different bacterial strains that competing with Anammox bacteria for NH_4^+-N caused a greater consumption of NH_4^+-N than NO_2^--N. Feeding the reactors with only NH_4^+-N resulted in a progressive reduction of NO_2^--N concentration due to a dilution effect as well as consumption by Anammox bacteria. Subsequently, to prevent further NO_2^-N

accumulation, from day 87 the molar ratio between NO_2^--N and NH_4^+-N was changed from 1.32 to 0.76, by adding in the influent solution 35 mg/L of NO_2^--N instead of 60 mg/L. In AcS reactor the concentrations of NH_4^+-N in the effluent fluctuated between 2 to 30 mg/L for the first 117 days, whereas, from day 117 it showed a decreasing trend (with values ranged between 0.1 and 5.1 mg/L) that was explained as an effect of an increase of the number and consequently the activity of Anammox bacteria. As a consequence of the good performance of the system, from day 128 the concentration of NH_4^+-N in the influent was furthermore increased from 46 to 58 mg/L and accordingly to a molar ratio of 0.86 was also increased the concentration of NO_2^--N up to 50 mg/L. Since the AcS reactor at this new operating conditions showed a removal efficiency approximately of 90%, the molar ratio between NO_2^--N and NH_4^+-N at day 141 was increased from 0.86 to 1 by adding to the influent solution, 58 mg/L of NO_2^--N instead of 50 mg/L, with the aim of making the molar ratio between the nitrogen based compounds progressively equal to the stoichiometric value of 1.32. From day 141, the nitrogen removal efficiency decreased to approximately 70%, presumably due to the increased NLR (0.03 kgN m^3 d^{-1}) and the short time given to the Anammox bacteria to get adapted to this new high load level. The nitrate production was relatively low during the whole experiment fluctuating between 2 and 26 mg/L. In the last 50 d it was noticed a significant decrease with values lower than 15 mg/L. This result was a proof of the successful enrichment process for the Anammox biomass in the sludge collected from the WWTP of Nola.

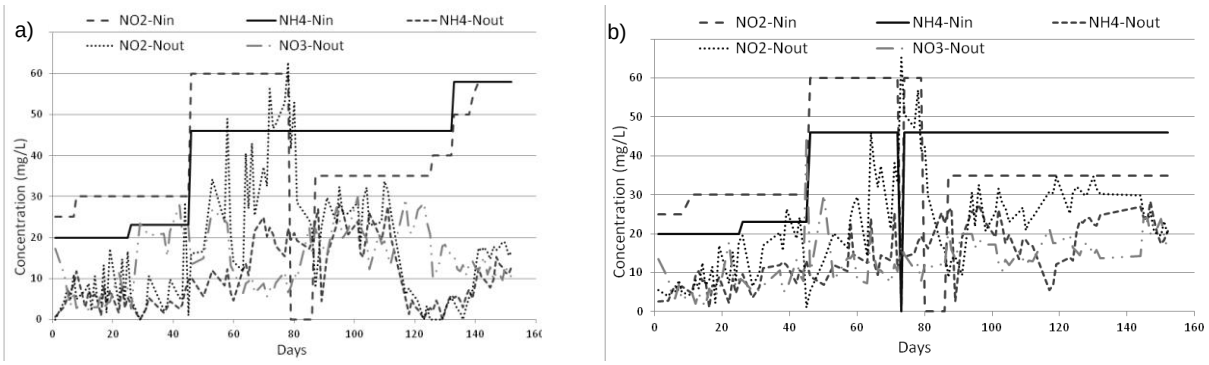

Figure 5.1: Evolution of N-based compounds during Anammox biomass enrichment: a) AcS reactor; b) AnS reactor

Reactor AnS did not show the same experimental results. From day120, the reactor showed anexcessive accumulation of NH_4^+-N, NO_2^-N and NO_3^--N, and therefore, in contrast to what was made with AcS reactor, the concentrations of NH_4^+-N and NO_2^--N in the feeding solution

were not changed and the molar ratio of 0.76 was maintained. From day 144 the concentration in the effluent of N-based compounds started to show a decreasing trend that was a proof of a good level of the Anammox activity, that was achieved almost 30 days after the AcS. This result proves that activated sludge from a denitrification tank is better than anaerobic sludge to develop Anammox biomass.

5.3.2 Partial nitritation-Anammox (CANON type) system reactors handling and performance

The process of the one reactor partial nitritation/Anammox is shown in Fig. 5.2. Both reactors were initially fed with an ammonium concentration in the influent of 5 mg/L NH_4^+-N that was gradually increased up to 20 mg/L NH_4^+-N on day 40. In the first period of operation, the reactors performed an ammonium removal of around 50% with a production of nitrates around 40% of the ammonium consumed in BRS1 and 30% in BRS2. In both reactors the nitrates production was actually higher but not much than the theoretical production that is 26% of the ammonium consumed. Feeding the reactors with a higher ammonium concentration (i.e. 30 mg/L) from day 50 with the aim of setting the condition for the removal of a high concentration of ammonium, it was noticed an accumulation of nitrate in the system up to a concentration of 60 mg/L NO_3^--N at day 86. This event was likely due to a faster growth of the nitrite-oxidizing bacteria (NOB) than Anammox biomass. To prevent the excessive production of nitrates the operating conditions were changed with the aim of limiting the growth of NOB and simultaneously increasing the number of Anammox. To achieve this purpose the oxygenation of reactors was previously reduced by turning off the micro pore air diffusers on day 90 because oxygen inhibits Anammox bacteria and a low concentration of O_2 limits the nitrate production. This operation resulted in a slight decrease of nitrate concentration but not enough compared to the efficiency level expected from Eq. (1). Therefore the mixing system was changed from a magnetic stirrer to a mechanical stirrer from day 107. This change in the mixing system favours the formation of granules of sludge, which usually containmoreAnammox bacteria in the inner part whereas the external part is mainly composed of AOB and NOB due to the oxygen gradient (Kindaichi et al., 2007). With this configuration the O_2 dissolved in the bulk inhibits less the Anammox activity because the bacteria living in the inner part of the granule have limited contact with O_2. To further moderate the nitrate production, the concentration of alkalinity in the feeding solutionwas reduced from 1 g/L to 177 mg/L from day 130 because AOB and NOB bacteria need a higher

amount (e.g. 1.98 mol of HCO_3^- per mol of $NH4^+$ converted for AOB and 0.5 mol of HCO_3^- per mol of NO_2^- converted for NOB) of inorganic carbon (IC) compared with Anammox bacteria that require only 0.066 mol of HCO_3^- per mol of NH_4^+-N consumed (Kimura et al., 2011). From day 163, the alkalinity was further lowered to 150 mg/L where a positive decrease of NO_3^--N concentration up to 20 mg/L was observed, presumably resulted from the granular structure as well as the decreased alkalinity. Simultaneously the reactors experienced an unexpected decrease in ammonium removal. This may be due to the granular configuration of Anammox sludge, which hampers the availability of IC to Anammox bacteria as IC has to diffuse into the granule to be used by Anammox bacteria. Therefore AOB and NOB, which compete for IC with Anammox bacteria, are favoured as they are located on the surface of the granules and have easier access to IC. To study this effect the two reactors were fed from day 200 with a different alkalinity concentration: in BRS1 the alkalinity was gradually increased, initially up to a valueof 69mg/L at day204, and then up to 200 mg/L at day 207 whereas in BRS2 it was gradually decreased up to 36 mg/L at day 187. The higher level of alkalinity in BRS1 resulted in an increase of the ammonium removal efficiency as well as nitrate production, whereas the lower level of alkalinity in BRS2 resulted in a further reduction of ammonium removal efficiency and the presence of nitrite in the effluent. This result proves that a low amount of IC affects more the activity of Anammox bacteria than AOB and NOB, contrary to the stoichiometry of the reactions, i.e. ammonium oxidation by AOB, nitrite oxidation by NOB and anaerobic ammonium oxidation by Anammox bacteria.

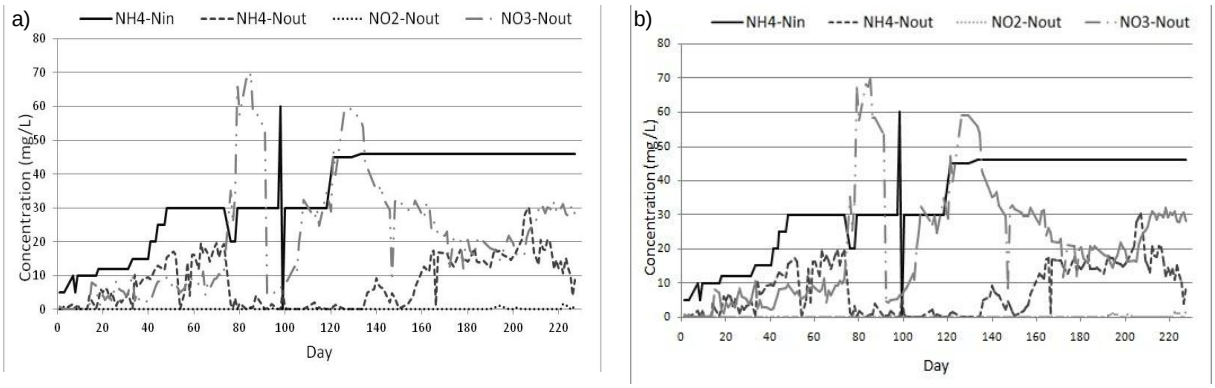

Figure 5.2: Performance of the Partial nitritation-Anammox: a) BRS1 reactor; b) BRS2 reactor

5.4 Conclusions

The research presented in this report has showed that the growth of Anammox biomass from wastewater treatment sludge as well as from the sludge of an anaerobic digester treating organic solids is possible even if very slow: actually after 160 days the enrichment process resulted in a partial growth of the Anammox biomass. In terms of enrichment performances the sludge from the denitrification tank of a WWTP was superior to the sludge from an anaerobic digester since the development of Anammox biomass was faster. Moreover the research showed the critical aspects concerning the managementof thecombined process of partial nitritation-Anammox (CANON-type system). Several attempts by changing the operating conditions were made with the aim of favoring the growth of Anammox rather than AOB and NOB, but no one gave the expected results. This outcome proves that it is difficult to manage in the same reactor the coexistence of different biomasses that compete for the same substrate and need different environmental conditions to grow.

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Chapter 6

General discussion and future perspective

Chapter 6 General discussion and future perspective

Similar to a good organization that requires efficient board of directives and human resource management, knowledge on the cooperation of microorganisms and their living environment is essential for scientists in environment biotechnology. The concept of microbial resource management (MRM) was proposed by Verstraete et al. (2007) which attempted to provide a decision making tool in steering the microbial capabilities associated to complex microbial communities (Read et al. 2011). MRM achieved success in the application in various environment and microorganisms (Read et al. 2011). In Section 1 of this chapter, key questions addressed from MRM will be introduced for the application of Anammox. In Section 2, position of the works in this dissertation will be discussed. In Section 3, future research of particular interest will be proposed.

6.1 MRM framework of Anammox research

6.1.1 The microbial "black box"

In order to steer the specific natural processes into manageable system performing enhanced microbial activity, which is known as "bioreactor", the following key questions are addressed by MRM:

a) Who is there?

Despite the slow growth of Anammox bacteria which hindered their isolation, efforts have been invested from worldwide science communities to reveal the taxonomies and ecophysiologies of Anammox. Molecular techniques provide strong tools to characterize Anammox bacteria till species level, among which FISH was the first and most used technique, followed by DGGE and real time PCR (Zhang and Liu 2014). To date, six genus and 19 species (candidatus) have been identified and submitted to the National Center for Biotechnology Information (NCBI) data base (Fig. 6.1) (Ali and Okabe 2015). All of them belong to the phylum *planctomycetes* which was earlier determined by (Strous et al. 1999).

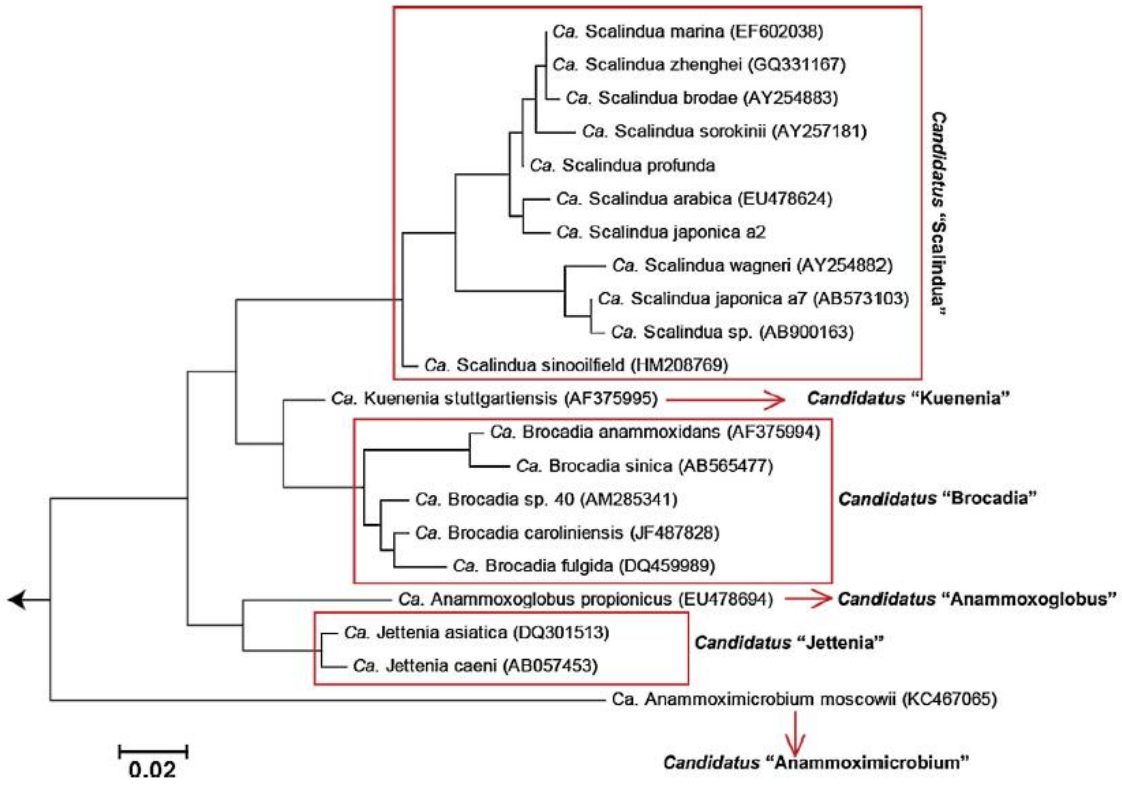

Fig. 6.1 Microbial diversity of Anammox bacteria (Ali and Okabe 2015).

It was speculated that the global distribution of Anammox bacteria is determined by salinity (Sonthiphand et al. 2014). For example, the genus *Scalindua* is only found in marine environment while others have different sensitivities or tolerance to salinity (Ali and Okabe 2015). This is of special interest in the selection of species and operation strategies in treating wastewater with high salinity (Liu et al. 2014). Other factors such as sulfide (Jin et al. 2013) and temperature (Daverey et al. 2015) also influence the distribution of Anammox bacteria.

b) Who is doing what?

Functions of the Anammox bacteria in natural or engineered environment are generally determined by the closed loop of nitrogen compounds as well as other metabolic products. Besides their key function of anaerobic ammonium oxidation, some species are able to perform other activities under certain conditions. For example, *Candidatus Anammoxoglobus propionicus* and *Candidatus Brocadia fulgida* could perform denitrification with propionate and acetate as electron donor (Guven et al. 2005; Kartal et al. 2007). Growth independent nitrogen conversion was observed under low SRT (Monballiu et al. 2013).

c) Who is doing what with whom?

The sustainable Anammox activity requires continuous supply of substrates, i.e. nitrite, ammonium and inorganic carbon. Simultaneously, it must be protected against dissolved oxygen and other inhibitory compounds. As mentioned previously, nitrogen mostly exists as NH_4^+ in wastewater. Nitrite is unstable by product from nitrification. Thus Anammox process is only feasible when coexisted microorganisms "cross feed" the Anammox bacteria with ammonium and nitrite with stoichiometric molar ratio of 1:1.146. AerAOB is the provider of nitrite for Anammox which could either coexist in single reactor system or in separate SHARON reactor. However, Anammox biomasses are mostly seeded from conventional sludge or natural habitats. Therefore it is almost inevitable that undesirable NOB and heterotrophic denitrifiers coexist with AerAOB and Anammox. On the other hand, the stoichiometrically maximum nitrogen removal through partial nitritation/Anammox pathway is 89%, leaving nitrate in the effluent. Thus denitrifiers are necessary either in the same reactor or separately to remove the residual nitrate.

The above three questions compose the major bricks of MRM. The functioning of a "healthy" microbial system is determined by the optimization of available energy, which is a result of cooperation between microorganisms. Thus the following three additional questions are addressed as "missing links" in MRM (Verstraete et al. 2007).

d) What is the minimum ΔG to switch to another partner or metabolism?

Switch of metabolisms happens when bacteria are under unfavourable condition to optimize the use of energy as survival strategy. This requires the activation of the functional gene as well as a more negative ΔG. However, knowledge on the switch of partner and metabolism with respect to the minimum ΔG is very limited. Rather conditions at broad scale are available. For example, organotrophic denitrification from Anammox bacteria occurred when the influent C/N ratio was less than 0.5 gCOD/gN. At this ratio, denitrifiers are outcompeted by Anammox for acetate (Winkler et al. 2012).

e) How important is cross feeding between species and what is the prevalent transport mechanism between the partners?

Anammox bacteria require cross feeding of nitrite from AOB and/or inorganic carbon source from coexisting heterotrophs. In the case of SNAD process, Anammox also cross feed denitrifiers for nitrate (Chen et al. 2009). Thus cross feeding is essential in Anammox based nitrogen removal systems. Transport is more efficient in granular and attached growth biofilm

configuration due to juxta position of the cells (Vlaeminck and Verstraete 2009). Transport through diffusion is also possible when Anammox bacteria formed granules or biofilms and AOB suspended in bulk (Figueroa et al. 2012). In the latter case the washout of NOB is crucial because NOB has higher affinity to nitrite.

f) How important is the architectural configuration of cells in flocs, granules and biofilms

Anammox bacteria have been reported to spontaneously form granules or attach to reactor wall (Monballiu et al. 2013). Cell aggregation is believed to be a bacterial physiology which is mediated by the production of EPS. Structures of Anammox granules are found either as homogeneously distributed (Ni et al. 2010) or layered (Vazquez-Padin et al. 2010; Vlaeminck et al. 2010; Volcke et al. 2010). In the latter case, Anammox composed the centre and AOB formed the rim, which is favourable for its anaerobic feature.

6.1.2 Engineer's design and system output

Depending on the influent characteristics and the desired output of the system, engineers must decide the configuration of the reactors as well as operation strategy and monitoring parameters (Vlaeminck et al. 2012). First of all is the **choice of reactor configuration and seeding sludge**. SBR is most frequently selected to cultivate Anammox granular sludge. Attached growth biofilm configurations are proved to be feasible, including MBBR, rotating biocontactor (RBC) and fixed bed reactor. Also the selection of one-reactor or two-reactor systems shall be considered which is dependent on the seeding sludge as well as the space limitation (Jaroszynski and Oleszkiewicz 2011). Enrichment of Anammox biomass from activated sludge, anaerobic granular sludge and sediments have been reported as feasible (Chamchoi and Nitisoravut 2007; Hu et al. 2013; Shen et al. 2012; Wang et al. 2011). Secondly engineers must design the **operation strategy**, including feeding/discharging regime, aeration, mixing, volumetric exchange ratio (VER), HRT, SBR cycle, rotation speed for RBC, etc. Among all these parameters, aeration and mixing may decide the shear stress of the biomass which affects the aggregate formation. Feeding/discharging regime and HRT decide the substrate load of the reactor. Those parameters are subject to modification according to the status of the reactor. Finally it is important to select the **monitoring parameters**. Generally the monitored parameters are: pH, DO, temperature, influent/effluent

nitrogen compounds, MLVSS, alkalinity, salinity and microbial diversity. Those parameters give feedback to the operation strategy.

The main desired output of the designed system include: (i) high nitrogen removal efficiency; (ii) accelerated enrichment and fast start-up; (iii) formation of granules or biofilms and (iv) high process stability. The output performances are indicated by the monitored parameters and give feedbacks to the operation strategy. Fig. 6.2 incorporates all the input/output parameters and the questions addressed by MRM, which attempts to cover all the aspects of research in Anammox based wastewater treatment.

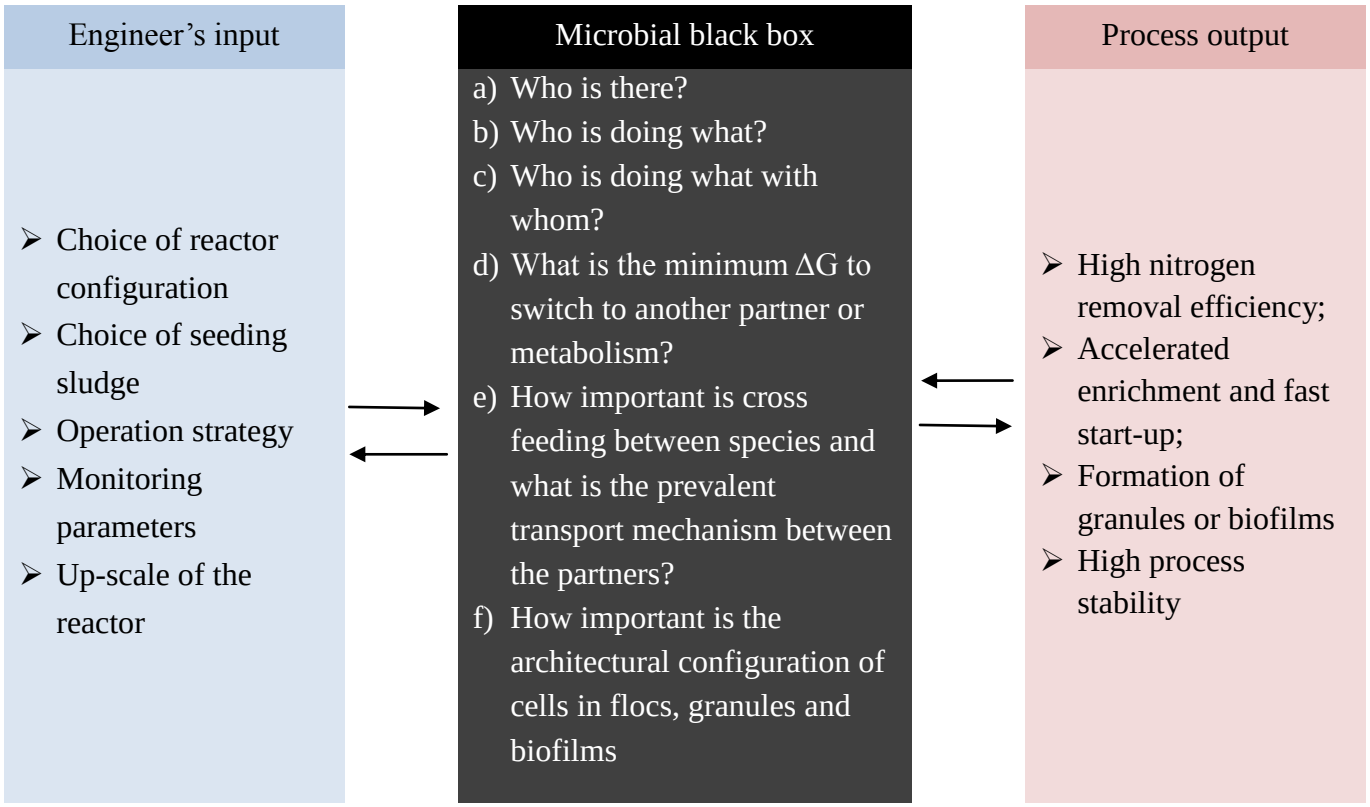

Fig. 6.2 MRM framework for Anammox process design (summarized and modified from Vlaeminck et al. (2012))

6.2 Position of this study in MRM framework

In this thesis, a series of studies have been conducted aiming at the main MRM output of "high nitrogen removal" and "accelerated enrichment" as indicated in Fig. 6.2. The enrichment of Anammox biomass in anaerobic SBR was investigated in detail which covered different aspects of the MRM framework for Anammox process (Chapter 3-4). Test of one-

reactor partial nitritation/Anammox (CANON-type) was attempted but the expected output was not achieved (Chapter 5).

Engineer's input was studied in Chapter 3. The "choice of reactor" and "monitoring parameter" was kept as constant. The same SBR configuration was constantly used and the monitored parameters were pH, DO, influent/effluent nitrogen compounds, MLVSS and alkalinity throughout all the tests. The investigated variables are the type of seeding sludge and operation strategy (HRT 2 days vs. 4 days; DO 1 mgO_2/L vs < 0.2 mgO_2/L). The monitored physico-chemical parameters served as first hand information for the evaluation of enrichment status and nitrogen removal performance. By analyzing those parameters, the following operational observation and conclusion were drawn:

- denitrification sludge is the most suitable seeding sludge for Anammox enrichment which resulted in a highest total nitrogen removal and shortest enrichment period;
- a shorter HRT or higher selection is preferred during the initial phase of the enrichment;
- DO control is of crucial importance over other control parameters to suppress the persisting AOB and NOB;
- Restoration of Anammox activity is possible after inhibited by high level of free ammonia and nitrite.

To reveal the dynamics of the "microbial black box", molecular technique PCR-DGGE was performed on different time of the enrichment period (Chapter 3). Quantitative and qualitative EPS analysis was conducted aiming at establishing the correlation between EPS characteristics and process parameters (Chapter 4). Also a general review on the role of EPS in bioaggregation was provided in Chapter 2 in which information on Anammox granule are included. The main conclusions are summarized as follows:

- dissimilarities between DGGE fingerprints of sludge samples from different time gave evidence of the evolution of microbial diversity, which is consistent with the changing process parameters;
- quantitative measurement of EPS components is related to the general microbial activity of the reactor. But the reliability of the results are affected by the methods of extraction and the possible overlapping between protein and polysaccharide;

- in 3D-EEMs from EPS samples from different time, EPS from sludge containing more Anammox bacteria more frequently possess two separate peaks in Zone I and II according to the delineation by Chen et al. (2003);
- UV chromatograms after SEC showed increase in both the intensity and number of peaks, indicating that more diverse molecular structure occurred as a result of Anammox enrichment. Fluorescence SEC chromatograms of all selected ex/em wavelength couple showed similar shape with differences in intensity;
- increase of hydrophobicity was observed during the enrichment period, presumably due to the aggregation of Anammox bacteria;
- Anammox granules either have homogeneous distribution of Anammox cells over the granule or layered structure in which Anammox bacteria compose the centre and AOB form the rim;
- hydrophobicity was believed to be the main force driving the aggregation of Anammox cells.

6.3 Future perspective

Over the past two decades, research and application of Anammox process experienced exponential growth. For example, the number of articles published per year on Anammox process grew from 2 in 1995 to 119 in 2012; topics on microbial diversity grew from 2 in 1998 to 38 in 2011 (Zhang and Liu 2014). The reveal of metabolic pathway and intercellular structure is of paramount importance and interest, both in the study of global nitrogen turnover and in the application of the process. At mean time, the first full scale Anammox installation was put in operation in 2002 and till now there are 114 full scale plants in the world. The volume of the plant ranges from 70 m^3 to 142,000 m^3 (Ali and Okabe 2015). Despite its huge progress and success, there are challenges which still need to be overcome by both engineers and scientists. The main challenges are: (i) slow growth of the bacteria which results in difficulties in isolation of pure culture as well as long start-up time; (ii) out selection of coexisting bacteria competing for substrates, especially NOB and (iii) application limited only on wastewater with low C/N ratio.

6.3.1 Effect of coexisting bacteria

Anammox biomasses are mostly enriched from conventional wastewater sludge or natural compartments such as marine sediments. Thus Anammox do live with other partners to form an ecosystem performing various niches. Although in earlier time the two-stage SHARON-Anammox was extensively studied and applied, the single-stage partial nitritation/Anammox gradually became the main stream, in which 90% of the full scale WWTPs belong to (Ali and Okabe 2015). Not only because the single-stage systems leave less cost and footprint, but also because the enrichment process in two-stage systems could not ensure the full exclusion of the undesired microorganisms thus could not lead to simpler operation. For example, NOB could survive under very low DO (0.5 mgO_2/L) and easily colonize the aggregate surface. The inhibitory FA threshold for NOB is 3 mgNH_3-N/L which is close to that for Anammox (5 mgNH_3-N/L) (De Clippeleir et al. 2013). It was mentioned in Chapter 3 that reactivation of NOB frequently occurred during the enrichment process when the total nitrogen removal deteriorates. It was thus proposed to be served as an indicator of the reactor performance. In practical, there could be two options to reduce the negative effect of NOB. One is to wash out the NOB at higher temperature and low HRT (SHARON process) from activated sludge (van Dongen et al. 2001). In this way the two-reactor system applies. The other is to suppress the NOB activity through substrate (FA) inhibition (Vlaeminck et al. 2009). This may lead to further challenge in maintaining the process stability. Future study could consider mixing different seeding sludges containing no NOB for the enrichment of Anammox biomass, e.g anaerobic granular sludge (Tang et al. 2010) and the nitritation sludge obtained from SHARON process.

From Chapter 2, it could be found that aerobic granules mostly have anaerobic and heterotrophic core. Also the mechanisms of aerobic granulation sometimes involve the initiation by filamentous bacteria. These factors might indicate the role of heterotrophs in the aggregation of Anammox bacteria. In the first phase of Anammox enrichment, denitrification was the prevailing process in the reactor. Heterotrophs utilize organic material from microbial activity such as the production of EPS and SMP and cell lysis. The presence of denitrifiers in Anammox reactor has both advantages and disadvantages. Since the single-stage process converts about ¼ of the ammonium into nitrate, post treatment on the removal of this part of nitrate is necessary to meet the standard. Denitrification could be performed within one reactor with partial nitritation/Anammox, which is named as “simultaneous partial nitrification, Anammox and denitrification (SNAD)” process (Wang et al. 2010). It was expected that the SNAD process could result in 95% COD removal and 85% nitrogen

removal (Ali and Okabe 2015). However the SNAD process faces more difficulties in process control due to the inclusion of one more major microbial activity.

Due to the limitation of influent C/N ratio, the application of Anammox reactor in mainstream application is limited. The majority of the currently running full scale plants were operated for the treatment of industrial wastewater (Lackner et al. 2014). If Anammox process is applied for mainstream wastewater treatment, additional pretreatment is needed to reduce the organic load. Also post treatment is necessary to removal the residual nitrate. However, to fulfil these requirements, it may compromise the benefit of reducing footprint. One feasible option is to modify the existing WWTP performing conventional activated sludge process. Taking the example of a WWTP consisting of anaerobic/anoxic/aerobic MBR, organic load could be largely reduced as it was in the anaerobic UASB reactor, pre-denitrification could also be used prior to the main partial nitritation/Anammox treatment, the aeration tank will be replaced by the single-stage partial nitritation/Anammox MBR, where solid/liquid separation could also be achieved.

6.3.2 Future research on EPS

The high investment of EPS production for autotrophic bacteria indicates its necessity in cell survival. First of all, EPS serve as glue material which facilitates cell aggregation. Second, it determines the intercellular distance and substrate diffusivity, presumably facilitating cross feeding. Third, the characteristics of EPS show high correlation with process performance (Chapter 2). However, considering the scarcity of Anammox sludge as well as its specific feature of slow growth, the method of EPS extraction and characterization shall differ from other conventional sludge. The currently available solubilization based EPS extraction methods all require large amount of concentrated sludge. After extraction, the sludge could not be recovered. Considering the long time for cultivation, which mainly resulted from its slow growth, frequent sampling and extraction for lab-scale or pilot-scale reactors are not economically practical. Furthermore, unlike other gram-positive and gram-negative bacteria, the Anammox cells contain no peptidoglycan. Thus mild extraction method of EPS from Anammox sludge shall be preferred to avoid high degree of cell lysis.

As an alternative to the commonly used photometric determination of EPS components, *in situ* visualization of sludge after selective staining could be a proper way to quantify different EPS components. The method provides information on the spatial distribution of different EPS components in the aggregates. Since the method require negligible amount of cells, it could be used as regular analysis as indicator of process performance. Furthermore, sampling from different parts of the reactor is possible to enable a higher representativeness. SEC coupled with fluorescence and UV detection gives information about the molecular weight distribution of the macromolecules. It is interesting to extract and purify the specific fraction and conduct further protein and/or polysaccharide structural analysis.

A final remark on the perspective of EPS study on Anammox bacteria is the extracellular enzyme. It was believed by different authors that protein compose major part of Anammox EPS. However, most studies on extracellular protein focused on its structural role in maintaining the EPS matrix. As extracellular enzymes were believed to be able to hydrolyse some organics (Yu et al. 2007; Zhang et al. 2015), it is of interest to discover its specific enzymatic activity especially in the case of layered structural granules.

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Curriculum Vitae

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Education

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PhD in Environmental Technology, Erasmus Mundus program of ETECOS3

Thesis title: *Anammox process in wastewater treatment: biomass enrichment and evolution of EPS characteristics*

Promoter: Prof. Dr. Giovanni Esposito

Research on Anammox enrichment process conducted in Laboratory of Sanitary Engineering, University of Naples Federico II, Italy

Microbial analysis was performed in Laboratory of Microbiology, University of Naples Federico II, Italy

Characterization of extracellular polymeric substances (EPS) was performed in University of Limoges, France.

Sept 2006 – Sept 2008

M.Sc in Environmental Sanitation, University of Ghent (Belgium)

Specialization: biological wastewater treatment and microbial ecology

Thesis Title: *Enhanced anaerobic digestion by inoculation with compost*

Promoter: Prof. Dr. ir. Willy Verstraete

Sept 2001 – Jul 2005

B.Sc in Environmental Science, Donghua University (Shanghai, China)

Specialization: wastewater treatment

Thesis Title: *Study of polyacrylamide grafted to sodium alginate copolymer flocculant*

Promoter: Prof. Dr. Zhou Meihua

Professional experience

Jan 2011- Dec 2011

Forest Carbon Expert at Conservation International (US based NGO), Beijing

Responsibility: Quantification of carbon sequestration through forestation

May 2010 – Dec 2010

Project Manager at MGM International, Beijing

Responsibility: Development and feasibility study of projects under

clean development mechanism (CDM)

Feb 2009 – May 2010 **GHG Auditor (expert) at TÜV Nord, Shanghai**

Responsibility: Validation and verification of CDM projects

Publications

Ding, Z., Bourven, I., Guibaud, G., van Hullebusch, E., Panico, A., Pirozzi, F. and Esposito, G. (2015) Role of extracellular polymeric substances (EPS) production in bioaggregation: application to wastewater treatment. Applied Microbiology and Biotechnology, 99(23): 9883-9905

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